2013

The Phylogenetics of Tachinidae (insecta: Diptera) with an Emphasis on Subfamily Structure

Daniel J. Davis

Wright State University

THE PHYLOGENETIC RELATIONSHIPS OF

TACHINIDAE (INSECTA: DIPTERA)

WITH A FOCUS ON SUBFAMILY STRUCTURE

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

DANIEL J DAVIS
B.S., Wright State University, 2010

2012
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

December 13, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Daniel J Davis ENTITLED The phylogenetic relationships of Tachinidae (Insecta: Diptera) with a focus on subfamily structure BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

_____
John O. Stireman III, Ph.D.
Thesis Director

_____
David Goldstein, Ph.D., Chair
Department of Biological Sciences
College of Science and Mathematics

Committee on Final Examination

_____
John O. Stireman III, Ph.D.

_____
Jeffrey L. Peters, Ph.D.

_____
Donald F. Cipollini, Ph.D.

_____
Andrew Hsu, Ph.D.
Dean, Graduate School

ABSTRACT

Davis, Daniel J. M.S., Department of Biological Sciences, Wright State University, 2012. Phylogenetic relationships of Tachinidae (Insecta: Diptera) with a focus on subfamily structure.

The parasitoid flies of the Tachinidae family are an important and diverse (>10,000 species) lineage of insects. However, tachinids are not well studied partially due to their confusing classification and taxonomy. DNA sequences were obtained from twenty tribal representatives of Tachinidae, along with eight outgroups in order to phylogenetically reconstruct the superfamilial, subfamilial and tribal relationships of Tachinidae. Seven gene regions of six genes (18S, 28S, COI, CAD, Ef1a, and TPI) were sequenced for each taxon (6214 bp total). Both maximum likelihood and Bayesian methods were used to infer phylogenies. The Sarcophagidae and Oestridae were usually reconstructed as monophyletic. Calliphoridae was paraphyletic with *Pollenia* typically being sister to Tachinidae. The Rhinophoridae were found embedded within an otherwise monophyletic Tachinidae, a unique finding. Subfamilies of Tachinidae were generally related in a (Tachininae + Exoristinae) + (Phasiinae (Dexiinae)) manner. The problematic Tachininae genera *Strongygaster* (Strongygasterini) and *Ceracia* (Acemyini) were placed into their original subfamilies with high confidence. These findings led to a new hypothesis about a slow evolution into the parasitoid habit.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

INTRODUCTION

Flies of the family Tachinidae (Insecta: Diptera) are ecologically and economically important due to their parasitoid lifestyle on other insects. Like other parasitoids, the larva of a tachinid develops inside of a living insect host and then kills it in order to reach adulthood. Tachinids attack a wide range of arthropod hosts including caterpillars (Lepidoptera), true bugs (Hemiptera), centipedes (Chilopoda), and spiders (Arachnida) (Wood 1987; Stireman et al., 2006). Tachinids also have a wide range of host attack strategies including laying larvae directly on the host, actively seeking out their host in the larval stage, and laying tiny eggs that the host ingests. Tachinids can be voracious parasitoids, accounting for up to 80% mortality of other insects (Boetner et al., 2000). Since the females of some species can lay up to 4,000 eggs over their lifetime (Belshaw, 1994), tachinids can be extremely effective at regulating populations of their hosts. For this reason tachinid flies have been extensively used as biological control agents against agricultural pest insects for over 100 years (Wood, 1987).

Tachinids have been widely used in managed biological control programs. For example, several species of tachinids have been introduced to control the gypsy moth (*Lymantria dispar*). Gypsy moth larvae are serious pests that defoliate hardwood trees and were introduced from Europe to the United States (Leihold et. al, 1992). This species defoliated 26 million acres of hardwood forest in a single major outbreak (1980-1982; McManus et al., 1992). Since the establishment of several tachinid enemy species on the gypsy moth, further

control efforts using pesticides have not been needed (Van Driesche et al., 2010). Other examples of successful biological control with tachinids include brown tailed moth, winter moth, sugarcane borers, mole crickets, and corn earworms (Grenier, 1998). These control efforts have reduced the need for pesticides and can be economical once establishment is achieved (Myers et al., 1998).

Although some control efforts using tachinids have been successful, many have been limited due to the lack of basic information about tachinids. In particular, the phylogenetic relationships of tachinids are poorly understood. The relationships among the 10,000 species of tachinids are ambiguous due to a high amount of morphological homoplasy throughout the family. This homoplasy creates identification problems and many scientists do not attempt to identify tachinids beyond the family level. This impedes managers of biological control programs when they are attempting to find suitable parasitoids for their project (Cooper et al., 2011). This is compounded by a lack of general knowledge about tachinid biology and their systematics. Systematic knowledge of tachinids is lacking due to a poor fossil record, the relative youth of the clade, morphological homoplasy, cryptic speciation, a high number of species, identification difficulties, and a lack of phylogenetic evidence (Crosskey, 1976; McAlpine, 1989; Stireman et al., 2006). However, we can now use molecular techniques to create a robust phylogeny that could fill in the knowledge gaps. A robust phylogeny also has the added benefits of bringing insight to the evolution of tachinids and their parasitoid lifestyle.

The focus of my research is using phylogenetics to construct a robust skeletal phylogeny for tachinids that will act as a basic framework for future research on this extremely large clade. By using phylogenetics, we can also look into the evolution of tachinids and the parasitoid lifestyle in general. The major goals of my research are:

- Use DNA sequence data to construct a robust phylogenetic framework for the family Tachinidae while emphasizing the subfamily structure.
- Place difficult taxa such as *Strongygaster* (Strongygasterini) and *Ceracia* (Acemyini) into appropriate subfamilies.
- Identify the sister-group to Tachinidae and their position within the superfamily Oestroidea.
- Use this framework to gain insight into the evolution of the parasitoid habit.

These goals will be achieved by using genetic sequencing and phylogenetic analytical techniques. Through these analyses, I will provide insights into the relationships within the Tachinidae and to advance our knowledge about the evolution of the parasitoid habit.

Tachinids represent a hyper-diverse lineage that may provide insight into the evolution of the parasitoid lifestyle. Tachinids are economically important as biological control agents against insect pests but a lack of basic knowledge of Tachinidae has created serious ecological problems. A robust phylogeny will clarify the relationships within tachinids as well as reveal insights into the evolution of the parasitoid habit. My research aims to resolve lingering questions about Tachinidae, their sister groups, and the evolution of the parasitoid habit.

Background

Family Tachinidae

The family Tachinidae is partially defined by the fact that all known species are arthropod parasitoids. A parasitoid possesses a unique lifestyle in which it develops within a living animal host, feeds off of it and eventually kills it in order to achieve adulthood. The parasitoid habit mostly occurs in Insecta, where they make up 10% of all insect species (Eggleton and Belshaw, 1993). Besides the hymenopteran parasitoids, Tachinidae is the next largest family of parasitoids. The parasitoid lifestyle confers several advantages including protection from direct predation, a readily available food source, and decreased food competition. In order to utilize the host, the adult parasitoid may paralyze the host (idiobiont) so the juveniles can feed on it, or the parasitoid may not paralyze the host to allow it to continue feeding and growing (koinobiont). Parasitoids may develop either outside (ectoparasitoid) or inside (endoparasitoid) the host. More than one egg from different parasitoids may occupy the same host (superparasitoid) and some parasitoids may use another parasitoid as a host (hyperparasitoid) (Godfray, 1994). Tachinids are obligate koinobiont endoparasitoids which may superparasitize some hosts. Although some tachinids do have piercing ovipositors, they do not paralyze their host because they lack venom glands that are common in hymenopteran parasitoids. Although no known tachinids are hyperparasitoids (Stireman et. al, 2006) they may be subject to extensive hyperparasitism by wasps (Kellogg et al., 2003).

The parasitoid lifestyle has evolved several times in a variety of lineages. By far the most diverse and well-studied group of parasitoids are the parasitic wasps in the order Hymenoptera. These parasitoids can use their long, piercing ovipositors to lay eggs on or within hosts that may be difficult to reach. The majority of hymenopteran parasitoids may be evolutionarily traced back to a single origin with the development of the piercing ovipositor (Rasnitsyn, 1988). Therefore, evaluating the evolution of the parasitoid habit in hymenopterans has limited value (Feener and Brown, 1997). Outside of the parasitic hymenopterans, Diptera (true flies) is the next largest order containing parasitoids with Tachinidae being the largest parasitoid family. Diptera contains at least 22, up to perhaps 100, different origins of the parasitoid habit (Eggleton and Belshaw, 1993; Wiegmann et al., 2011). One of these origins occurs within the tachinid lineage due to sarcophagy being the plesiomorphic condition (McAlpine, 1989). Given their great ecological and evolutionary success, along with the wide variety of reproductive strategies they possess, the family Tachinidae is an excellent group for studying the evolution of the parasitoid habit. The evolution of the parasitoid habit may be the key innovation that has allowed tachinids to become one of the most rapidly radiating lineages of flies.

The tachinids are the second largest dipteran family with as many as 10,000 described species distributed worldwide (Irwin et al., 2003). The entire family is composed of parasitoids but they have a wide range of lifestyles, host use patterns and reproductive methods. Tachinids are typically nectar or honeydew feeders as adults and are effective pollinators, especially at higher

altitudes where other insects become less abundant (Coombs and Dold, 2011; Kearns, 1992; Wood, 1987). Tachinids use a wide variety of arthropod hosts but typically parasitize phytophagous larvae of Lepidoptera and Coleoptera or nymphs of Hemiptera and Orthoptera. Tachinids have not been known to parasitize eggs or pupa, but 5-10% of tachinids are known to attack adult stages of other insects (Stireman et al., 2006). Many tachinids are considered to be generalists in their host selection but they may be more specialized than currently thought given the likelihood of cryptic species complexes (Feener & Brown, 1997; Smith et al., 2007). Many of the host species that are attacked by parasitoids have developed behaviors to counteract parasitoid attack such as evading the parasitoid or adjusting foraging behaviors (Gross, 1993; Singer and Stireman, 2003). Once the larva is inside of the host, it needs to evade the host's immune response. Tachinid larvae may either form a respiratory funnel by manipulating the host's encapsulation response or the tachinid may move to a region of the host where the host is incapable of encapsulating and killing it. This allows the host to continue to feed and grow while the tachinid larva is feeding inside the host. Once the larva has eaten and killed its host, it will pupate and develop into an adult (Stireman et al., 2006).

Although Tachinidae are almost exclusively koinobiont endoparasitoids (non-paralyzing, internal parasitoids), the method of oviposition varies widely among species. Tachinids may directly oviposit on a host or they may indirectly oviposit near a host. Tachinids may also lay incubated larvae covered by only a thin egg chorion (ovolarvipary) instead of undeveloped eggs (ovipary). Some

tachinids lay tiny, so-called “microtype” eggs on foliage that the host ingests. Tachinids as a group do not possess an ancestral piercing ovipositor like hymenopteran parasitoids. However, piercing structures composed of modified terminal sternites have evolved in several lineages (notably the Phasiinae and Blondeliini) to assist in oviposition. Once a host has been parasitized, the tachinid larvae will grow inside and feed upon the host until it has been killed (Stireman et al., 2006). This parasitoid lifestyle is a desirable characteristic when identifying biological control agents.

Tachinidae as Biological Control Agents

Tachinids have been used extensively in biological control programs against insect pests of economic significance (Van Driesche et al., 2011). Applied biological control attempts to introduce an invasive species’ natural enemies from their home range onto the invasive population. For invasive insects, parasitoids are often used as natural enemies because of a higher specificity than predators. The parasitoid tachinids can be a significant cause of mortality (>80%) for some herbivorous insects, especially Lepidoptera (Boettner et al, 2000). Since a single tachinid female can lay several thousand eggs (Belshaw,1994) they have the opportunity to kill more of their hosts than the typical predator can. For these reasons, tachinids have been extensively used in applied biological control.

Tachinids have been used in over 100 biological control programs throughout the United States and the world. Beginning in 1905, several tachinid species were released to control the forest pests *Lymantria dispar* (gypsy moth)

and *Nygmia phaeorrhoea* (brown tailed moth) that devastated timber crops in North America (Grenier, 1988). The forest pest *Operophthera brumata* (winter moth) was controlled using the tachinid *Cyzenis albicans* in western Oregon (Kimberling et al., 1986). Five species of tachinids were successfully used in the United States to control the European corn borer between 1920 and 1937 (Bake et. al, 1949). Throughout the world, various tachinids have been successful in controlling sugar cane borers (De Bach, 1974). Tachinids have also been used to control the coconut moth, mole cricket, and corn earworm (Grenier, 1988). Recently, introductions of the tachinid *Lixadmontia franki* have been attempted against the bromeliad attacking weevil *Metamasisus callizona* to prevent widespread losses of rare bromeliads throughout Florida (Cooper et al., 2011). The high mortality rates that tachinids inflict have been instrumental in controlling invasive pest insects, despite having mixed results.

Although there have been some great successes using tachinids as biological control, there have also been many failures. Many of the tachinid releases have not resulted in establishment for a myriad of reasons. These reasons include spatial and temporal variation between climates, failure to get tachinids to reproduce in the wild, competition from predators or hyperparasitoids, failure of the population to overwinter, or not enough genetic diversity to maintain the population (Grenier, 1988). Besides establishment, another potential problem of biological control programs using tachinids is non-target effects (Louda et al., 2003). The generalist nature of some tachinids was used as reasoning to release them. These tachinids can overwinter on non-target

host and persist even if the target host population decreases. However, the release of tachinids as biological control can result in the decline of native species that are not the target of the biological control (Boettner et al., 2000). The cost of extensive tachinid releases may be prohibitive. However, once a tachinid population becomes established, it becomes a part of the natural ecosystem and may have lasting benefits. Using a long term outlook on biological programs using tachinids, the benefits often outweigh the costs if establishment is achieved (Myers et.al., 1998). Establishment is difficult and is complicated by a lack of knowledge of the biology, ecology, and phylogeny of tachinids. A better understanding of tachinid biology and phylogeny can help mitigate the complications of using them in applied biological control programs.

Tachinid family relationships

Tachinids belong to the large superfamily Oestroidea. Like other calyptrate flies, members of the Oestroidea possess calypters which act as small winglets underneath their wings, located over their rear spiracles. Flies in the Oestroidea also possess a unique row of bristles on their thoracic meron, above their hind pair of legs. Members of superfamily Oestroidea have distinct wing venation including the vein M1 forwardly deflected (bent) and vein A1 not attaining wing margin, but there are exceptions. The tachinids possess these characteristics along with five other primary families. Other member of Oestroidea include the families Sarcophagidae (flesh flies), Rhinophoridae (isopod parasitoids), Calliphoridae (blow flies/cluster flies), and Oestridae (bot flies) (McAlpine, 1989).

The superfamily Oestroidea also contains Mystacinobiidae, a monotypic family of a rare bat parasite that will not be further discussed.

The tachinids can be easily identified by the presence of a well developed and sclerotized subscutellum which appears as a pronounced protrusion underneath the scutellum on the rear of the thorax. The rhinophorids are isopod parasitoids that possess a weakly developed, half-membranous subscutellum, and may be closely related to tachinids (Pape, 1986; Pape, 2010). Oestrids generally have stout bodies with weakly developed mouthparts and are all internal parasites of vertebrates. The sarcophagids can be most easily identified by 3 black stripes dorsally across the thorax. Most sarcophagids are sarcophagous although some are parasites (Miltogramminae) and parasitoids (*Sarcophaga* and *Helicobia*: Shewell, 1987; Eggleton and Belshaw, 1992). The calliphorids are typically carrion and dung feeders. However, the calliphorids have such varying characteristics that they are now thought to be a non-monophyletic group (Rognes, 1997). None of the oestrids or sarcophagids posses a subscutellum. A few calliphorids posses a subscutellum but it is not as well developed as the swollen, convex subscutellum of the tachinids. Given the wide variety of morphological differences between families and the paraphyly of the calliphorids, discussions regarding the relationships within superfamily Oestroidea have not led to a consensus phylogeny for the group (McAlpine, 1989; Wood, 1987; Yeates and Wiegmann, 1999). A phylogenetic analysis may unravel some of these complications.

Tachinid Systematics

The original classification systems of tachinids focused on the external morphological features of adults and extensive use of generic-level classifications. From the 1880's to the 1940's, C.H.T. Townsend classified 1555 new species, mostly in monotypic genera (Arnaud, 1958). Townsend extensively used chaetotaxy to classify individual species based on the arrangement and size of bristles on the body. However, this classification scheme led to extensive morphological homoplasy, classifying tachinids into multiple families while placing some sarcophagids within Tachinidae. In order to make better sense of the tachinids, scientists such as Herting (1960) and Wood (1987) began to classify tachinids based on reproductive habits, egg laying habits, and male genitalia. The majority of recent higher-level taxonomic work on tachinids has focused on these areas.

Tachinids have three distinct methods of laying eggs including oviparity, ovolarviparity and micro-type eggs. In the oviparity condition the eggs are laid with practically no embryonic development while the eggs of the ovolarviparous condition are laid with well-developed larvae. In the third method of egg laying, called microtype, tachinid females lay tiny eggs on a substrate (typically a leaf) that the host ingests. The ingested eggs then hatch in the gut and burrow into the host. (Stireman et al, 2006). Although these various egg laying conditions have been used to help decipher generic and tribal associations within the tachinids, there is still debate about their evolution through the subfamily levels. The oviparous state is considered the ancestral (pleisomorphic) condition in the

Oestroidea superfamily (McAlpine, 1989). However, there is some argument about how many times the ovolarviparous state developed within Tachinidae, complicating the phylogeny of Tachinidae with more homoplasy. Given these complications, researchers have been trying to unravel the phylogeny of Tachinids using various traits including eggs, larvae, pupa, terminalia, host use, and genetics (Stireman et al., 2006).

Most researchers now recognize four subfamilies of tachinids and yet only one has a definitive morphological distinction. The other subfamilies are based on host use and differing reproductive methods. All members of the subfamily Dexiinae have a hinged aedeagus (or phallus). Phasiinae are united by their parasitism on Hemipterans. The subfamilies Tachininae and Exoristinae are difficult to distinguish between morphologically. Most Tachininae have a uterus and lay eggs that are ready to hatch (Wood, 1987), but this is also found in many Exoristinae. The Exoristinae generally have a setose prosterum but other tachinids may also have this feature. Several authors have postulated relationships within and between the subfamilies. Herting (1984) proposed a (Phasiinae + Exoristinae) + (Tachininae + Dexiinae) scheme based on egg morphology while Shima (1989) proposed a (Tachininae + Exoristinae) + (Phasiinae + Dexiinae) scheme. However, a consensus of tachinid subfamily relationships has not been reached.

Although generic-level classifications are reasonably well defined, tribal and subfamily placements and classifications have remained contentious. This includes tribes like Strongygasterini and Acemyini (which includes the genus

Ceracia). The tribe Strongygasterini morphologically appears to belong to the Phasiinae, but it attacks beetles and ants instead of Hemiptera. Strongygasterini was originally classified as a phasiine (Herting, 1984) but it was later moved to the Tachininae due to its host use (O'Hara and Wood, 2004). The tribe Acemyini has also been moved recently from the Exoristinae to the Tachininae. Tschorsnig (1985) analyzed the genitalia of Acemyini and found it to be very similar to Strongygasterini. This evidence, along with the parasitism on grasshoppers by Acemyini, prompted Wood to move Acemyini to the Tachininae along with Strongygasterini. These are just a few examples of the revisions and reclassifications that have been occurring within the family Tachinidae. Phylogenetic analysis may be able to help clarify the taxonomic placements of these tribes, their subfamilies, and the family Tachinidae as a whole.

Molecular Phylogenetics of Tachinids

Given the difficulties of using morphology to understand the phylogeny of Oestroidea in general, and tachinids specifically, researchers have been turning to genetic analysis. Several higher level phylogenetic analyses of the Diptera and various superfamilies have been performed recently. Analyses of the order Diptera as a whole (Wiegmann et al., 2011), the subsection Calyptratae (Kutty et al., 2010; Nirmala and Zurovec, 2001), and the superfamily Muscoidea (Kutty et al, 2008) all have included Tachinidae in their analysis. Phylogenetic investigations have also been performed on related groups of calyptrate Diptera including Scathophagidae (Kutty et al., 2007) and Hippoboscoidea (Peterson et al., 2007). In these molecular analyses, relationships within the Oestroidea have

been inconclusive thus far with relatively weak support (<50% bootstrap support) for many branches (Kutty et al., 2010). Since Tachinidae were not the focal taxa in these studies, the tachinid sample sizes have been small in each case.

Very little genetic analysis has been conducted directly on tachinids. The subfamily Exoristinae has been the target of two studies due to the different developmental and reproductive strategies within the family. Stireman (2002) found evidence for the monophyly of Tachinidae and Exoristinae using the 28S and EF1α genes. Stireman's data supported the subfamily classifications based on reproductive habits and genitalia that was proposed by Herting (1984) and Wood (1987). Stireman also concluded that the taxa possessing microtype eggs were not monophyletic and that this reproductive strategy may have changed at least three times within the tachinids. Tachi and Shima (2009) analyzed the evolutionary history of female oviposition strategies on a phylogeny of Exoristinae they reconstructed using the 16S, 18S, 28S, and white genes. They also observed subfamily structure that reflected reproductive habits. However, unlike Stireman, Tachi and Shima found the micro type egg bearing taxa to be monophyletic. These two genetic analyses focused upon the Exoristinae, providing inconclusive data about the overall subfamily structure of tachinids and the placement of tachinids within their superfamily. Questions revolving around the evolution of tachinids and their parasitoid lifestyle can be answered by focusing on tachinids and their relatives. Since some members of Oestroidea are parasitoids while others are not, the parasitoid lifestyle must have evolved within this superfamily. Including the other members of the superfamily in phylogenetic

analyses may reveal insights into the evolution of the parasitoid lifestyle within this clade.

New Methods of Phylogenetic Reconstruction

Technology has made enormous leaps in the last several decades, making phylogenetic work both cheaper and easier. High throughput sequencing and cheaper primer construction have drastically decreased the price of genetic data. This has allowed researchers to develop more and better primer sets to aid in phylogenetic reconstruction. The advent of faster computer processors and cheaper memory has allowed researchers to conduct more advanced analyses when they are attempting to reconstruct phylogenies. The time it takes to complete a computationally intensive analysis has drastically decreased. New software tools such as GARLI, MrBayes, and BEAST have been developed to use these advances in computer processing and aid in phylogenetic reconstructions (Zwickl, 2006; Ronquist and Huelsenbeck, 2003; Drummond et al., 2012). These new methods of phylogenetic reconstruction have been instrumental for recent taxonomic work and will be able to aid in resolving relationships within the tachinids.

One of the primary ways to create phylogenies is using maximum likelihood analysis. Maximum likelihood calculates the probability that the observed data would fit the proposed model of evolution (Swofford et al., 1996). However, a maximum likelihood analysis can only test for one model of evolution at a time, often chosen using a model test. Consequently, this means that a simple maximum likelihood analysis should not be used for more than one gene

since each gene may be evolving under differing selective pressure. More recently, the ability to simultaneously use multiple models of evolution for different data sets was incorporated into the GARLI progam (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006). The GARLI algorithm uses a stochastic genetic approach to maximize the likelihood of the observed data. This method allows the user to concatenate all of the genetic data to be able to get a clearer picture of the phylogeny under the maximum likelihood criterion.

Alternatively to likelihood methods, Bayesian methods differ by their use of a prior distribution of parameters (Felsenstein, 2004). Bayesian inference alone is not very useful for phylogenetic analysis unless the Markov chain Monte Carlo (MCMC) algorithm is used. The MCMC algorithm takes the prior tree, modifies it, checks it against your data, accepts or rejects it, and if it is accepted, it then modifies that tree again and restarts the process. The chain is typically stopped once convergence is reached and the tree no longer drastically changes (Huelsenbeck et al., 2001). To use Bayesian inference with MCMC, programs such as MrBayes (Ronquist and Huelsenbeck, 2003) and BEAST (Drummond et al., 2012) have been developed. MrBayes has become the standard program to use for the Bayesian analysis of genetic data to create phylogenetic species trees. The program BEAST (Bayesian Evolutionary Analysis by Sampling Trees) uses MCMC but can use coalescence-based estimations to generate a species tree from multiple different gene trees. This means that BEAST does not condition itself from a single tree topology like MrBayes. Instead, BEAST weighs each gene tree proportionally to its posterior probability to create rooted trees

with a time scale. Although BEAST can help overcome the problems of incomplete lineage sorting by allowing the use of multiple genes, it may not be necessary for deeper phylogenies. These new, computationally intensive analyses have allowed researchers to better utilize genetic data in order to infer phylogenies and will be vital in understanding the Tachinidae

Objectives and Hypotheses

The primary objectives of this study revolve around establishing the evolutionary relationships of Tachinidae by using phylogenetic analysis of molecular sequence data. With this data I established three primary objectives for this study. First I used DNA sequence data to construct a robust phylogenetic framework for the family Tachinidae while emphasizing the subfamily structure. In this process, I also discovered new subfamily placements for difficult taxa. Secondly, I attempted to identify the sister-group to Tachinidae and evaluate the family's placement within superfamily Oestroidea. Finally, I used the phylogenetic reconstruction to gain a better understanding of the evolution of the parasitoid habit in the tachinids. The Tachinidae are one of the most taxonomically complex families of all Diptera (Crosskey, 1980) and phylogenetic analysis may be the best way to answer these questions.

The first goal focused upon providing a framework for the Tachinidae family while examining their subfamily structure. Current theories of the subfamily relationships include Herting's (1984) (Phasiinae + Exoristinae) + (Tachininae + Dexiinae) and Shima's (1989) (Phasiinae + Dexiinae) + (Exoristinae + Tachininae). However, subfamily structure is open to interpretation due to

differing opinions on critically evolved traits including egg thickness or egg laying method. There are also lingering questions about the placement of taxa such as *Strongygaster* (Strongygasterini) and *Ceracia* (Acemyini), which do not fit well into any specific subfamily. Phylogenetic analysis can help resolve these subfamily relationships.

The second goal aimed to find the sister group of Tachinidae. Rhinophorids have been moved between different families by various researchers, but they are now considered their own family. Traditionally the rhinophorids are considered the sister group to the tachinids. Rhinophoridae and Tachinidae share a common parasitism of arthropods and possession of subscutellum. Also, Rhinophorid females lay unincubated eggs and their first-instar larvae have two mandibles (absent in tachinids), both traits thought to be plesiomorphic (McAlpine, 1989). Rhinophorid larvae possess a unique cephalopharyngeal apparatus distinguishing them from other families of Oestroidea (Pape, 2010). Although the parasitic oestrids or one of the parasitoid calliphorids (*Pollenia* for example) could be the sister group, I hypothesized that the rhinophorids will be the sister group to Tachinidae.

The final goal was to examine the evolution of the parasitoid habit within the tachinids. It is thought that the plesiomorphic condition of Oestroidea is saprophagous, similar with the other calyptrates (McAlpine, 1989). The parasitoid habit must have arisen from this ancestral state somewhere within the superfamily. By analyzing the relationships within the superfamily I may be able to assess when the parasitoid habit arose, specifically in relation to the tachinids.

The parasitoid habit may have evolved before the split between the rhinophorids and the tachinids. Elucidating the evolutionary conditions of the parasitoid habit could be applied to other parasitoid lineages.

MATERIALS AND METHODS

Experimental Design

In order to meet the objectives of this study, several design characteristics had to be considered. First, a wide range of taxa needed to be selected throughout the tachinid family and superfamily Oestroidea. Secondly, I needed to amplify as many nuclear genes as possible. Finally, I needed to use the most modern analytical techniques in order to assess phylogenetic relationships. These principles directed how the study was performed.

In order to achieve good taxa coverage throughout the large Tachinidae family, I chose five genera, each from different tribes, for each of the four subfamilies. If possible I chose the representative taxa from that tribe. For example I chose the genus *Winthemia* from tribe Winthemiini and the genus *Uramya* from tribe Uramyini. Two taxa from each of the families of Calliphoridae, Sarcophagidae, and Oestridae were chosen. Each of these taxa are in different subfamily groups within their respective family. The only common isopod parasitoid in the United States (*Melanophora roralis*) was used for the family Rhinophoridae for most of the analyses. Although calliphorids are thought to be paraphyletic (Rognes, 1997), they are included as an essential part of the superfamily. The two calliphorids chosen were the common green bottle fly (*Lucilia sericata*) and *Pollenia* sp. (an earthworm parasitoid) in order to have a wide coverage of the calliphorids. Finally, *Musca domestica* (common house fly) was chosen as the far outgroup due to the availability of DNA sequences in GenBank and its placement within the calyptrate flies but outside of Oestroidea.

GenBank was used to gather sequences for *Musca domestica* and various other taxa in order to increase the gene coverage for the analyses (Tables 1 and 2). Overall this provided a total of 28 taxa (plus 3 additional rhinophorids for a single analysis) covering five families of Oestroidea and the four subfamilies of Tachinidae.

Over 100 primer pairs were tested for DNA amplification with only 7 pairs eventually working within our study group. The nuclear genes that worked were 18S, 28S, CAD (two sections), EF1α, and TPI. The mitochondrial bar coding gene COI was also used (Table 4). The nuclear genes PGD, AATS1, and period had some success but the amplification was inconsistent. Genes that did not work include white, wingless, and RNA polymerase II. Of the genes that did work, 18S and 28S create an RNA product used in the 18S and 28S subunits of ribosomes. As with most ribosomal genes, there are multiple copies of 18S and 28S within the genome. The CAD gene encodes for three enzymes important in the beginning steps of pyrimidine biosynthesis. Two sections of the CAD gene were successfully amplified. EF1α (elongation factor one) is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome and also has several copies on different chromosomes. However, the primers that were used were specific enough to amplify only one copy of EF1α. The TPI gene encodes for the protein triose phosphate isomerase. The mitochondrial COI gene encodes for the first subunit of cytochrome oxidase and is also called the bar-coding gene (Pruitt et al., 2012). These seven genes provided the sequence data of 6214 total base

pairs. A total of 173 sequences were obtained for 93% gene coverage (Tables 1 and 2) for the phylogenetic reconstruction of Tachinidae.

Laboratory Methods

The specimens were collected by either netting or trapping with Malaise traps. Upon capture, the specimens had one to three legs removed and placed into 95% ethanol. The specimens were then identified and pinned. The legs were stored in 95% ethanol at -20°C until they could be used as tissue for DNA extraction. The specimens were collected from across North America with a majority of specimens coming from the temperate woodland surrounding Wright State University in Dayton, Ohio. The specimens for *Cuterebra* sp. (oestrid) and *Phasia* sp. (tachinid) were provided by Dr. James O'Hara of the Canadian National Collection (Agriculture and Agri-Food Canada).

The removed legs provided the tissue necessary for the DNA extraction. The DNA samples with ID numbers less than T458 were extracted by a previous researcher while all other DNA samples were extracted by myself (Tables 1 and 2). All samples were extracted with Puregene Core Kit A (Qiagen Inc.) using a slightly modified manufacture's protocol. Each specimen was frozen in liquid nitrogen and crushed before cell lysis solution (200µL) was added. After incubation (65°C for 15min.) the sample was treated with 1.0µL of RNase A solution and then incubated again at 37°C for 15-60 minutes. The proteins were precipitated by using 70µL of protein precipitation solution followed by centrifugation at 12,000 rpm for 3 minutes before removal of the proteins. The DNA carrying supernatant was treated with 200µL of isopropanol in order to

precipitate the DNA. After centrifugation (12,000rpm for 5min.) and removal of the supernatant, the DNA pellet was washed with 200µL of 70% ethanol and then allowed to dry. After rehydrating the DNA in 100µL of DNA hydration solution overnight, the final result was stored at -20°C to be later used in PCR amplification.

DNA was amplified using touchdown PCR and was verified with gel electrophoresis. Touchdown PCR was used to increase the likelihood of amplifying the target sequence while reducing the probability of amplifying non-target DNA. This is helpful when you have fairly specific binding sites but multiple divergent copies of the gene such as EF1α (Palumbi, 1996).The annealing temperatures for the touchdown process went from 50°C, to 48°C then to 45°C (Table 5). Once the DNA was extracted, primers for the target gene were used to run a 10µL test PCR reaction. If the gene was successfully amplified, then a 30µL reaction was performed to create enough DNA for sequencing. The resulting reaction typically created 80-120ng of DNA for sequencing.

Sequencing was performed by the University of Arizona Genetics Core using an 3730XL DNA Analyzer (Applied Biosystems, Inc.). A total of four 96 well-plates were used. The Arizona Research Labs sequencing facility at the University of Arizona performed PCR product clean up for the first, second, and fourth plates. PCR cleanup for the third plate was performed using EXO SAP (Applied Biosystems, Inc.) at Wright State University. For each gene, both the forward and reverse sequences were amplified in order to get a longer total sequence. The sequences were added together later during the editing process.

Once the DNA was sequenced at the University of Arizona, the electropherograms were downloaded and then edited. The software CodonCode Aligner (CodonCode Corporation) was used to read, edit, and initially align the electropherograms. Both the forward and reverse sequences were aligned and then later combined together. Alignment was performed in two ways. For the ribosomal genes 18S and 28S, the secondary structure was predicted using RNA Fold (Lorenz et. al, 2011), creating an alignment constraint file. The default parameters for RNA fold were used including minimum free energy, partition function, and avoiding isolated base pairs. The program RNAsalsa (Stocsits et. al, 2009) was used to maximally predict the likely alignment for the sequences by based on the alignment constraint file. The default settings were used for RNAsalsa. All of the other genes (CAD, COI, EF1α, and TPI) were aligned in MEGA5 (Tamura et al., 2011) using the Clustal W algorithm. Each gene was then further manually aligned to remove unnecessary gaps. Each of the 172 sequences were visually checked and edited at least three times throughout the alignment process. The aligned sequences were then analyzed using maximum likelihood and Bayesian methods.

Analytical Methods

After sequence editing, maximum likelihood analyses were performed. Initially each gene was analyzed using maximum likelihood in MEGA 5 (Tamura et. al, 2011). A model test was used to identify the best nucleotide substitution model for each gene (Table 3). Although several option variations were attempted during the analysis, the following settings were primarily used: 1000

bootstraps, five gamma categories (when gamma distribution was used), partial deletion, 95% site cutoff score, all codon positions used, NNI Heuristic method, and an automatically generated initial tree. This missing data treatment for the CAD gene was set to use all sites due to the large amount of missing information. The program GARLI (Zwickl, 2006) was able to analyze a combined concatenated data set under the maximum likelihood criterion. The default configuration file was used except for the following: availablememory = 2048, logevery = 50, saveevery = 500, searchreps = 2, and bootstraps = 500. All resultant trees where then rooted with *Musca domestica*. All aesthetic changes to trees were performed in MEGA 5.

Three different Bayesian analyses were performed using MrBayes (Ronquist and Huelsenbeck, 2003) under differing conditions. Individual data sets for each gene were combined into a single nexus file for the analysis including data for the 28 primary taxa and all seven gene sections. Each run of MrBayes used similar parameters (Appendix I) with appropriate gene partitions, 3 million generations and a 50% burn in. Stationarity was typically reached well before one million generations. MrBayes was first run on the combined data set. A second run was performed with Tachinidae constrained as a monophyletic group. Finally, a third analysis was run using extra sequences from GenBank for the Rhinophoridae family. The added rhinophorids from GenBank (Kutty et. al, 2008; Kutty et al., 2010) included *Paykullia maculata*, *Stevenia hertingi*, and *Stevenia atramentaria* (Table 2). These trees were also aesthetically modified using

MEGA 5. These three analyses provided some of the most robust results of this study.

An alternative to MrBayes is BEAST (Drummond et al., 2012), which uses coalescence methods to estimate an overall species tree. Since individual gene trees may not reflect the species tree due to incomplete lineage sorting, coalescent methods can be used to infer phylogeny. BEAST attempts to bridge the gap of using Bayesian MCMC for phylogenetics and coalescent-based population genetics. BEAST applies the Metropolis-Hastings MCMC for a coalescense-based estimation of phylogeny by explicitly modeling the rate of molecular evolution on each branch of the tree. This allows BEAST to focus on calibrated phylogenies that contain a time-scale and to reduce complications of incomplete lineage sorting. The problems of incomplete lineage sorting typically do not affect phylogenies at the target taxonomic level. However, BEAST was still used in order to accomplish due diligence during this study. Similar to maximum likelihood, BEAST required a model test to identify the best substitution model. BEAST had fewer model options therefore the next best model was used (Table 3). Empirical base frequencies were used with a chain length of 100 million sampled every 10,000 chains. A lognormal relaxed clock was used while root height was set at 0.15 and the Yule process was set as the species tree prior. The resultant trees were annotated and then aesthetically modified using FigTree (Rambaut, 2012).

RESULTS

Maximum Likelihood

Each individual gene evaluated with maximum likelihood (Figures 1 - 6) showed wildly varying results. Only 13 of the 122 bootstrap values were above 50% while there were 14 polytomies throughout the 6 gene trees. None of the individual gene trees recovered Tachinidae as a monophyletic group although EF1α had only the rhinophorid outgroup *Melanophora roralis* placed in the tachinid family (Figure 5). The 18S, CAD and TPI genes were able to recover the Sarcophagidae as monophyletic (Figures 1, 4, and 6). The Oestridae was always paraphyletic while the family Calliphoridae was monophyletic for only the 28S gene (Figure 2). The rhinophorid *Melanophora roralis* had a tachinid as its sister taxon in 18S, 28S, and EF1α but not in COI (Figures 1, 2, 5, and 3). *Pollenia* was a sister taxon to a tachinid in 18S, COI, EF1α and TPI (Figures 1, 3, 5, and 6).

Due to the wildly differing results with the maximum likelihood analyses, information gained about subfamily structure was fairly minimal. No discernible subfamily structure can be seen with the 18S, EF1α or TPI gene trees (Figures 1, 4. 5, and 6). The 28S gene tree recovered most of the Dexiinae and Exoristinae into their respective clades (Figure 2). The COI tree was able to recover most of the Exoristinae and Phasiinae into different clades (Figure 3). With the alternate missing data treatment, the CAD tree was able to recover the core phasiine group (*Gymnosoma*, *Trichopoda*, and *Phasia*) as well as four of the five Exoristinae. The inconclusive results of the individual maximum likelihood trees prompted the use of other analytical methods such as GARLI (Zwickl, 2006).

The program GARLI was used to evaluate maximum likelihood after combining all of the data of the individual genes together. Although GARLI did not report bootstrap values under 50%, GARLI was able to recover a mostly monophyletic Tachinidae, Sarcophagidae, and Oestroidae (Figure 7). The calliphorid *Pollenia* appears to be the sister group of Tachinidae while *Lucilia* was the sister group of Oestridae. The rhinophorid *Melanophora* was embedded within the tachinids. Within the tachinids, the core groups of each subfamily were recovered. Within the tachinid family there is a basal split between the (Tachininae + Exoristinae) and the Phasiinae (Dexiinae). *Ceracia* appears within the Exoristinae as the sister to *Winthemia* (96% support). *Strongygaster* was embedded within the Phasiinae, basal to *Campylochaeta* and *Melanophora* (Rhinophoridae). The Phasiinae themselves were paraphyletic with a mostly monophyletic Dexiinae embedded within them. The dexiine *Camplylochaeta* appears within the phasiines but not within the Dexiinae.

MrBayes Analyses

Three differing analysis were performed using MrBayes, each one resulting in similar results. The first analysis only used the data that was used in the maximum likelihood analyses. In the phylogenetic reconstruction resulting from this analysis, there was an initial basal split between the Sarcophagidae and the (Oestridae + Tachinidae) group (Figure 8). The calliphorid *Lucilia sericata* was sister to the sarcophagids with the Sarcophagidae being monophyletic. Oestridae was recovered as monophyletic and sister to *Pollenia* + Tachinidae. The calliphorid *Pollenia* appeared as the sister group to tachinid with 99%

posterior probability. Tachinids were recovered as monophyletic with the exception of *Melanophora roralis* which is placed within the Dexiinae.

The initial Bayesian analysis recovered each of the four Tachinidae subfamilies to some degree. Within the tachinids, there is an initial split between the (Exoristinae + Tachininae) and the Phasiinae (Dexiinae) (Figure 8). The tachinine *Ceracia dentata* appears within the Exoristinae while the tachinine *Strongygaster* is placed within the Phasiinae. The Dexiinae are embedded within the Phasiinae although the Dexiinae *Voria ruralis* is outside of the core Dexiinae group. *Melanophora roralis* also appears within the Dexiinae with *Thelaira americana* as its sister group.

Due to the unexpected placement of *Melanophora roralis*, the analysis was rerun with a constraint that the Tachinidae were monophyletic. In the resultant tree, *Melanophora* was placed basal to all other Oestroidea (Figure 9). Two novel groupings between (Sarcophagidae + Oestroidea) and (*Pollenia* + Tachinidae) appeared with low support (45%). Oestridae and Sarcophagidae were each monophyletic with *Lucilia sericata* being basal to the sarcophagids. The calliphorid *Pollenia* was still reconstructed as the sister group to Tachinidae. Within the tachinids, the basal (Exoristinae + Tachininae) and (Phasiinae (Dexiinae)) split was recovered again. *Ceracia dentata* was placed within the Exoristinae and *Strongygaster* appeared within the Phasiinae as with the previous analysis. Finally, the Dexiinae became monophyletic by restricting Tachinidae to exclude *Melanophora roralis*.

The possibility of Rhinophoridae being a part of Tachinidae was further explored in a third Bayesian analysis by adding GenBank sequences of other rhinophorids into the analysis (Table 2). The constraint of a monophyletic Tachinidae was also removed. The results were similar to the initial analysis at the superfamily level (Figure 10). Sarcophagidae was monophyletic and basal to Oestridae and Tachinidae. *Pollenia* was the sister group to Tachinidae once again. Within the tachinids, there was an initial split between (Tachininae + Exoristinae) and (Rhinophoridae + (Phasiinae (Dexiinae)). Again, *Ceracia dentata* and *Strongygaster* were placed within Exoristinae and Phasiinae respectively. Excluding *Ceracia* and *Stongygaster*, the rhinophorids, Tachininae, Exoristinae, and Dexiinae were all monophyletic. However, most of the posterior probabilities were lowered in this analysis compared to the previous two.

Species tree analysis in BEAST

A very different result was recovered when using BEAST to analyze the data and infer a species tree. The species tree resulting from this analysis contains a basal split between most of the outgroups and Tachinidae (Figure 11). Both *Cuterebra* (Oestridae) and *Melanophora* (Rhinophoridae) are embedded within the tachinids. *Pollenia* is placed as the basal lineage of other outgroups with the paraphyletic sarcophagids, *Lucilia sericata* (calliphorid), and C*ephenemyia* (Oestridae). Within Tachinidae, the BEAST analysis split tachinids between the Dexiinae and everything else. As in previous analyses, *Melanophora* appears within the Dexiinae, but so does *Cylindromyia binotata* (Phasiinae). The second major clade contains most of the Tachininae,

Exoristinae, and Phasiinae. The Phasiinae were embedded within the Exoristinae, who themselves were embedded within the Tachininae. However, several placements did not correlate with the other analyses (MrBayes and GARLI). *Cuterebra* (a botfly) and *Catharosia* (Phasiinae) appeared within the Exoristinae. *Winthemia* (Exoristinae) and *Ceracia* (Tachininae) both appeared within the Phasiinae. However, the uniformly lower posterior probabilities indicate that the tree topology was not well supported.

DISCUSSION

The purpose of this research was to examine subfamily structure of the Tachinidae family, assess the sister-group to tachinids, and gain insights into the evolution of their parasitoid habit. These objectives were met by utilizing the most recent phylogenetic reconstruction methods. The results were highly varied, as to be expected with one of the most taxonomically complex families of all Diptera (Crosskey, 1980). However, significant insight can still be gained from this approach and this study can help lay the foundation for future systematic work on the Tachinidae family.

This study used seven gene regions to help reconstruct the phylogeny of tachinids with varying degrees of phylogenetic utility. The difficulty lay in trying to find consistent phylogenetic signal in such a varied group of genes. This study attempted to generate a more informative phylogeny by using a combination of quickly evolving genes (COI) and slowly evolving genes (18S and 28S). The most informative genes appeared to be CAD, COI, and TPI while 18S, 28S, and EF1α provided less information at our targeted taxonomic level. The most informative genes had slightly higher bootstrap support, were less likely to place outgroups within Tachinidae, and generated relationships that latter appeared on the combined analysis. However, these individual genes alone were not sufficient to draw reliable conclusions. All of the genes needed to be combined to gain better insight into the phylogenetic relationships of Tachinidae.

The wide variations in evolutionary rate among genes created a situation where the individual maximum likelihood analysis of each gene provided very low

support for the nodes. Due to this, the individual ML analyses were generally rejected in this study in favor of the more complex GARLI, MrBayes, and BEAST analysis that used the combined data set. The maximum likelihood results were useful during the course of this research, but the focus changed to the results of the combined analysis because they resulted in much more coherent and plausible phylogenies. This is primarily due to combined analysis being able to use the entire 6214 base pairs of information while the individual gene trees were much more limited. The combination of the faster evolving genes and slower evolving genes, along with simply more data, created a more complete and reliable picture of the tachinid phylogeny.

Among the combined analyses, GARLI and MrBayes had extremely similar results while the species tree reconstruction in BEAST proved to be less useful. The trees produced by GARLI had a similar structure to the trees produced by the MrBayes analysis except that most nodes had much weaker support. Any values less than 50% were not reported. GARLI also generated a unexpected clade within the Phasiinae that included *Campylochaeta* (Dexiinae), *Strongygaster*, and *Melanophora*. MrBayes provided some of the clearest results with very strong basal support for many clades. BEAST did provide information on tachinid phylogeny but many of the relationships were muddled at the more basal levels with extremely weak support (2-18% posterior probability). This is probably due to BEASTs' algorithm attempting to integrate across gene trees instead of relying on the combined data set as in MrBayes. Also, species tree methods are best used on shallow phylogenetic divergence, not the deep

divergences that this study targeted. The ability of GARLI, MrBayes, and BEAST to use a combined data set provided the greatest insight into the phylogeny of the Tachinids.

Relationships among the Oestroidea

The superfamily Oestroidea is composed of five major families including Sarcophagidae, Oestridae, Tachinidae, Calliphoridae, and Rhinophoridae. Of these groups there is some evidence for the monophyly of Sarcophagidae, Oestridae, Tachinidae, and Rhinophoridae based on the limited taxon sampling. The evidence also indicates that the calliphorids are paraphyletic and that the rhinophorids may be a clade of the tachinids. One of the calliphorids, *Pollenia*, may be the sister group to the tachinids and rhinophorids. Several general conclusions can be made regarding the monophyly and relationships among the families of Oestroidea even with the sparse taxonomic sampling of the families (especially in Calliphoridae). These findings coincide with current hypotheses about the monophyly of these families except for the rhinophorids.

Good evidence, both morphological (McAlpine, 1989) and genetic (Kutty et. al, 2010; Weigmann et al., 2011), already exists for the monophyly of Oestroidea as a whole. In this study the sarcophagids *Helicobia* and *Macronychia* were consistently recovered as a monophyletic clade throughout the study with high support (68%, 98%, 97%, and 97% in combined analyses; Figures 7-10). The sarcophagids were also recovered in three of the six locus-specific maximum likelihood trees, although with lower support (Figures 1, 4, and 6). The bot flies *Cephenemyia* and *Cuterebra* were also consistently recovered

as a monophyletic clade (67%, 96%, 99%, and 100%; Figures 7–10) even though they were never placed together in the individual gene trees. Species tree analyses in BEAST did not recover either the sarcophagids or oestrids as monophyletic.

Monophyly for the family Tachinidae is already well supported by molecular evidence (Stireman, 2002; Tachi and Shima, 2009). This study also found evidence for tachinid monophyly but with a few caveats. The rhinophorid *Melanophora* consistently appeared within an otherwise monophyletic Tachinidae. None of the individual gene trees using maximum likelihood recovered a wholly monophyletic Tachinidae. However, the combined analyses (Figures 7, 8, and 10) did recover Tachinidae as a clade with high support (98% and 72%) as long as *Melanophora* is included. The BEAST analysis weakly supported tachinid monophyly with both *Melanophora* and *Cuterebra* embedded within it. The tachinids are widely considered a monophyletic group and their (near) monophyly in the analyses provides more confidence in GARLI, MrBayes, and BEAST, which all use a concatenated data set.

The two calliphorid genera in this study were almost always paraphyletic. Only in the 28S maximum likelihood analysis did they create a monophyletic group. There is very good morphological evidence for the paraphyly of the calliphorids (Rognes, 1997) and the results of this study support it. This study used two completely different calliphorids, the coprophagous common green bottle fly (*Lucilia sericata*) and the earthworm parasitoid *Pollenia*. *Lucilia* was selected due to the fact that it is among the most common calliphorids. *Pollenia*

was chosen due to its unique parasitism of earthworms. The fact that *Pollenia* consistently appeared to be the sister group to the tachinids and rhinophorids was a surprising result.

One of the primary objectives of this study was to determine the sister group to Tachinidae. In most cases, the calliphorid genus *Pollenia* was the sister group of Tachinidae (Figures 1, 3, 5-10). This result is somewhat unexpected but not implausible due to the parasitoid habit of *Pollenia*. Rhinophorids were expected to be the sister group to tachinids based upon their parasitism of arthropods and the possession of a subscutellum. However, rhinophorids were embedded within the tachinids across most analyses with fairly strong support (Figures 7, 8, 10, and 11). It is possible that certain lineages of calliphorids are closely related to tachinids due to the paraphyly of calliphorids. Considering that the larval stage of *Pollenia* are host-seeking earthworm parasitoids, this is suggestive that *Pollenia* and possibly other, unsampled calliphorids may be the sister group to Tachinidae. However, due to the complications of the placement of the rhinophorids, a definitive answer continues to be elusive.

The phylogeny of Oestroidea is currently uncertain based on both morphology (McAlpine, 1989) and genetics (Kutty et al., 2010). Questions about the evolution of the parasitoid habit and problems with the paraphyly of the calliphorids continue to complicate the matter. The conflicting results of this study do not provide a resolution to these issues. However, as with the monophyly of the families, general conclusions can be made. The six individual gene trees that utilized maximum likelihood analysis did not produce any consensus about the

relationships within the superfamily Oestroidea. The results from GARLI (Figure 7) and the analysis using MrBayes unrestricted (Figures 8 and 10) produced similar results. These results produced a clade resembling a Sarcophagidae (Oestridae (*Pollenia* (Tachinidae (Rhinophoridae)))) scheme. The calliphorid *Lucilia* appeared basal to the oestrids in the GARLI results and basal to the sarcophagids (but not the rest of Oestroidea) in the MrBayes results. Due to limited taxon sampling, we cannot draw conclusions about the relationship between the unrepresented calliphorids and other members of Oestroidea. In these analyses it appears that some calliphorids (*Lucilia*) are more closely related to Sarcophagidae while others (*Pollenia*) are more closely related to Tachinidae. This coincides with other recent phylogenetic analyses performed on the calliphorids (Marinho et. al, 2012). The BEAST analysis produced a very different picture regarding the relationships within Oestroidea. BEAST produced a highly polyphyletic outgroup containing sarcophagids, oestrids, and calliphorids while producing a mostly monophyletic Tachinidae group that included the rhinophorid and the oestrid *Cuterebra*. BEAST provided extremely low support value for all of these basal relationships. The information provided by BEAST about the relationships within Oestroidea is limited due to the extreme paraphyly of the outgroups, as well as the inclusion of *Cuterebra* within the tachinids. However, due to the nearly congruent trees of GARLI and MrBayes regarding family relationships, there is evidence that superfamily Oestroidea follows a Sarcophagidae (Oestridae (*Pollenia* (Tachinidae (Rhinophoridae)))) relationship scheme.

Rhinophoridae

During the course of this study, the rhinophorids were repeatedly embedded within the tachinids. Although rhinophorids share many similar characteristics (subscutellum and obligate arthropod parasitism), their placement within the tachinids was surprising. Rhinophorids have historically been placed in several different families including Tachinidae, Calliphoridae, Sarcophagidae, and are currently within their own family (McAlpine 1987). Their half-membranous subscutellum (versus the fully sclerotized subscutellum of Tachinidae) and their parasitism of isopods (unique in insects) created problems for their phylogenetic placement within Oestroidea. Due to their external morphology, the rhinophorids were thought to be a part of the tachinids (Sabrosky and Arnaud, 1965) or a very close family (Crosskey, 1977). Members of Rhinophoridae lay unembryonated eggs near hosts, similar to the calliphorid *Pollenia* and many Tachinidae. However, calliphorids themselves are not monophyletic and require re-ranking (Wood, 1997). Such a comprehensive re-ranking may place some calliphorids near the rhinophorids and tachinids. Rhinophorids also share similar larval morphologies, including a distinct elongate anterior process of the pharyngeal sclerite (Rognes, 1986). The combination of these morphological dissimilarities with the other members of Oestroidea was thought sufficient evidence to warrant their promotion to the family level. However, due to the variable placement of the rhinophorids within the tachinid family during the Bayesian analysis, their placement needs to be reexamined.

The initial deep placement (Figure 8) of the rhinophorid *Melanophora* within a paraphyletic Dexiinae (which is defined by a morphological synapomorphy) using the Bayesian analysis created concern. Although the sister group to *Melanophora* was a tachinid in 3 of the 4 genes in maximum likelihood analyses, the rhinophorid was still expected to be outside of Tachinidae due their differences in morphology. The analysis was rerun with a constraint creating a monophyletic Tachinidae, thus making it impossible for *Melanophora* to be nested in Tachinidae. The resultant tree (Figure 9) placed the rhinophorids as the far outside group to all of Oestroidea, an unlikely result. The third analysis removed the constraint and added additional rhinophorid taxa from GenBank (Table 2). The extra taxa moved the rhinophorids outside of the Phasiinae (Dexiinae) clade but still within the tachinids (Figure 10).

The cause of *Melanophora's* placement so deep within Tachinidae may have been the lack of sequence data. *Melanophora roralis* did not have data for the TPI or CAD gene. These genes contained some of the strongest phylogenetic signal along with 2222 bps (35%) of the genetic data. With data for only 18S, 28S, COI, and Ef1a, the rhinophorids may have been incorrectly placed within the Dexiinae. After adding the additional rhinophorids, the analysis reduced the posterior probabilities of several nodes, including Tachinidae monophyly (from 98 to 72), probably due to a lack of gene coverage in the added rhinophorids. This is the first molecular evidence that rhinophorids may actually be a part of tachinids. If they are a part of Tachinidae, they are probably from a very early branching lineage of the Phasiinae, which are thought to contain

several basal tachinids. This could explain why they differ so greatly in morphology from other known tachinids. If the rhinophorids are not a part of the tachinids, then they are likely to be closely related, even being their sister group. However, with the lack of complete sequence data for the rhinophorids and sparse taxon sampling, no definitive conclusion can be reached about their placement.

Tachinid subfamily relationships

The subfamily structure of the Tachinidae is fairly well resolved in the combined data set analyses (Figures 7-11) but is severely lacking in the individual gene trees. Most analyses recovered a subfamily structure of (Exoristinae + Tachininae) + (Phasiinae (Dexiinae)) with the rhinophorids embedded within Dexiinae. This is similar to previously proposed subfamily groupings of Shima (1989) who proposed (Exoristinae + Tachininae) + (Phasiinae + Dexiinae). The GARLI and MrBayes analyses provided the clearest picture of subfamily structure. The BEAST analysis provided a much more muddied picture with much lower support for nearly every branch. The (Exoristinae + Tachininae) + (Phasiinae (Dexiinae) grouping does not hold any major conflicts with current tachinid knowledge (except Rhinophoridae).

The Phasiinae (Dexiinae) clade is very well supported by GARLI and MrBayes analyses once the addition of *Strongygaster* is considered. The monophyletic Dexiinae appears to be nested within the typically paraphyletic Phasiinae. A monophyletic Dexiinae is expected due to the shared synapomorphy of a hinged aedeagus (phallus). The paraphyletic Phasiinae is not

entirely unexpected due to the lack of synapomorphies within the clade (Wood and Zumbado, 2010). The Dexiinae were recovered in just a single gene tree (Figure 2) while the other gene trees have the Phasiinae and Dexiinae mixed throughout the rest of tachinids. GARLI was able to recover the Phasiinae (Dexiinae) clade although the support levels were less than 50% (Figure 7). Two of the MrBayes analyses (Figures 8 and 9) recovered the Phasiinae (Dexiinae) group with very strong support (93% and 98%). However, when the additional rhinophorids (Figure 10) were added, the posterior probability dropped significantly to 53%. With the additional rhinophorid taxa, MrBayes moved the Rhinophoridae outside of the Phasiinae (Dexiinae). Despite the shortcomings of the individual gene trees and BEAST reconstructions, the Phasiinae (Dexiinae) clade appears to be well supported in this study due to the relative strong results in trees inferred using GARLI and MrBayes.

This study produced strong support for the Exoristinae + Tachininae clade. These two subfamilies are similar morphologically so it is of little surprise that they were also similar genetically. This clade was recovered in the GARLI analysis and very well supported in MrBayes where it was consistently supported with 100% posterior probability. This clade was much less apparent in BEAST where the support values for every node were extremely weak and several Phasiinaes were embedded within the Exoristinae. However, due to the clades' recovery in GARLI and the consistently strong support with MrBayes, there is little doubt about the monophyly of the Exoristinae + Tachininae clade.

Both GARLI and MrBayes were able to recover a core group of Phasiinae that includes *Phasia*, *Trichopoda*, and *Gymnosoma* (Figures 7-10). These three genera were also recovered as a clade in the CAD and Ef1α gene trees. In the BEAST analysis the Phasiinae (including *Strongygaster*) were mixed with the other tachinids. *Cylindromyia* was typically placed either very close to or as a sister group to the Dexiinae. *Catharosia* was typically placed as basal to the rest of the Phasiinae. Among the more interesting results is the placement of *Strongygaster* within the Phasiinae. One of the goals of this study was to identify the subfamily of difficult taxa such as *Strongygaster*. This genus attacks beetles and ants, unlike the rest of the Phasiinae that attack hemipterans. Placement of *Strongygaster* with the phasiines had strong support (94%, 93%, and 86%) in the MrBayes analysis. GARLI also placed *Strongygaster* within the Phasiinae, albeit with lower support. *Strongygaster* also had a Phasiinae as its sister taxa in the 18S gene tree (Figure 1). It must be noted that *Strongygaster* was missing the MCAD, Ef1α, and TPI gene sections. However, such strong support and consistent placement within Phasiinae suggests that *Strongygaster* should be moved back to the Phasiinae despite their differing host use.

Although there were some discrepancies, the Dexiinae were typically recovered as a monophyletic group. This is expected due to their common synapomorphy. Four of the five Dexiinae were recovered as a monophyletic group in the GARLI analysis. The three MrBayes analyses were able to recover all of the Dexiinae as a monophyletic group even though *Melanophora* was embedded within it. After inclusion of additional rhinophorid taxa, the

Rhinophoridae moved outside of the Dexiinae. BEAST recovered four of the five Dexiinae as a single monophyletic clade even though the support was very weak. Overall there was strong evidence for the monophyly of the Dexiinae which supports the current thoughts about the subfamily.

The monophyly of Exoristinae was well supported with two internal clades. The first internal clade is comprised of *Tachinomyia*, *Lespesia*, *Hyphantrophaga*, and *Blondelia* while the second clade included *Winthemia* and *Ceracia*. The Tachininae *Ceracia* is strongly supported as a member of the Exoristinae with Winthemia as its sister group. *Ceracia* (tribe Acemyini) was originally classified as an exoristine but Tschorsnig (1985) identified similarities between the Acemyini and Strongygasterini in the male postabdomen. Since Strongygasterini was moved to the Tachininae, so was Acemyini (along with *Ceracia*). In light of this new genetic evidence, it appears that the male postabdominal similarities were homoplasious, just as Tschorsnig originally suggested. Further evidence of *Ceracia*'s inclusion in Exoristinae includes the similar way in which *Ceracia* and *Winthemia* deposit unembryonated eggs. Overall the Exoristinae forms a solid monophyletic group with strong support that is sister to the Tachininae.

The Tachininae formed a monophyletic group that included *Panzeria*, *Epalpus*, and *Siphona.* This group was the only group that had strong support in every single combined analysis (Figures 7-11). Of the five original Tachininae tribes included in this study, only these three remained within the subfamily. *Strongygaster* was consistently placed in the Phasiinae, its former classification. *Ceracia* consistently appears within the Exoristinae (its former classification).

Aside from the removal of *Strongygaster* and *Ceracia* from the Tachininae, this subfamily still had the highest monophyletic support of this entire study.

One of the primary objectives of this study to was to identify the underlying subfamily structure of Tachinidae using phylogenetics. From this data, strong support exists for the monophyly for three of the four groups. The tachinids form a subfamily structure of (Exoristinae + Tachinidae) + (Phasiinae (Dexiinae)). This is similar to Shima's (1989) subfamily scheme except for a paraphyletic Phasiinae. However, due to the incomplete sampling of this study and to keep tachinid classifications stable, it is suggested that the Phasiinae retain their full subfamily status. Reorganizing the Phasiinae into monophyletic clades lies outside the scope of this study. The genetic data supports current ideas about subfamily monophyly and gives insight into the tachinid phylogeny at the subfamily level.

Evolution of the Parasitoid Habit

One of the primary objectives of this study was to gain insight into the evolution of the parasitoid habit in the Tachinidae lineage. This study was able to recover a general superfamily structure of Sarcophagidae (Oestridae (*Pollenia* (Tachinidae (Rhinophoridae)))). From this scheme, two ideas are prevalent. First, the parasitoid habit probably evolved before the tachinids diverged from their sister group. Secondly, there appears to be a slow evolution into the parasitoid habit and not a sudden emergence of this lifestyle. These findings have implications beyond the superfamily that could be applied in other parasitoid lineages.

From these data it is fairly apparent that the parasitoid habit evolved before the divergence of tachinids. Even if rhinophorids are later found to be sister to the tachinids, the placement of *Pollenia* is highly suggestive that the parasitoid habit evolved before the divergence of Tachinidae. *Pollenia* is an earthworm parasitoid with host-seeking larvae and this lifestyle (or a similar one) probably led into the use of arthropods. Once the tachinid ancestor was able to aptly use arthropod hosts, they rapidly radiated into the over 10,000 species they are today. This radiation may have been extensive partly due to cascading speciation (Abrahamson and Blair, 2008). The key to the rapid radiation of the Tachinidae family may be the evolution of the parasitoid habit upon its arthropod host.

Within superfamily Oestroidea there appears to be a slow evolution into the parasitoid habit from a coprophagous or sarcophagus ancestor. If I consider my best supported phylogenies to be accurate (Figures 7, 8, 10), then it can be posited that a flesh feeding (sarcophagous) or perhaps coprophagous sarcophagid-like ancestor gave rise to the Oestridae. Somehow the lineage was able to overcome the immune response of its host in order to be able to live within its flesh, similar to an oestrid. This flesh living lineage may have given rise to a flesh living calliphorid that then evolved as a parasitoid that kills its host, similar to *Pollenia*. This then can give rise to a parasitoid that is able to utilize an arthropod. This arthropod parasitoid would then diverge into the current Rhinophoridae and Tachinidae lineages. The two novel adaptations in this hypothesis are the ability to avoid the immune response of the host (developed in

an Oestridae-like lineage, improved in *Pollenia*) and then utilizing an arthropod host (developed before the Tachinidae and Rhinophoridae divergence). This hypothesis of the evolution of the parasitoid habit within the Tachinidae lineage is supported by the results of this study. Although there are other parasitoid lineages within Oestroidea, this evolutionary pathway is one which the tachinids may have taken.

Future Directions

This research provides a solid foundation for future phylogenetic work with Tachinidae and their related families. Any future work examining the subfamily structure of tachinids would benefit by having additional taxa and more genes. Additional taxa could resolve some of the more complex relationships among the varying subfamilies and tribes. It is difficult to categorize 10,000 species by only 20 representatives as this study did with tachinids. Added taxa could clarify difficult relationships and increase support of the more basal relationships. This study's design of one genus per tribe was beneficial and could be useful in a future study. However, this design might not be possible if tribal associations are unclear. Adding additional genes to a similar study will also benefit any future work. Whenever more genes were added to the analyses they generated a clearer picture of the relationships. This corresponds with Maddison and Knowles (2006) in that the extra loci provided independent evidence of deeper relationships for deeper phylogenies. Some genes generated better information (CAD, COI, and TPI) than others (18S, 28S, EF1α) and this should affect the choices of which genes to use in the future. A researcher must also be wary of

the utility of the ribosomal genes due to their tendency to have some highly conserved regions and some rapidly evolving regions. Adding more taxa and more genes would benefit any type of future work on tachinids. Future research might include reclassifying the Phasiinae into monophyletic groups, tracing the evolution of the subscutellum through Oestroidea, or clarifying the position of the Rhinophoridae in relation to the Tachinids. With such a large and important group as Tachinidae, there is much work still left to do.

FIGURES

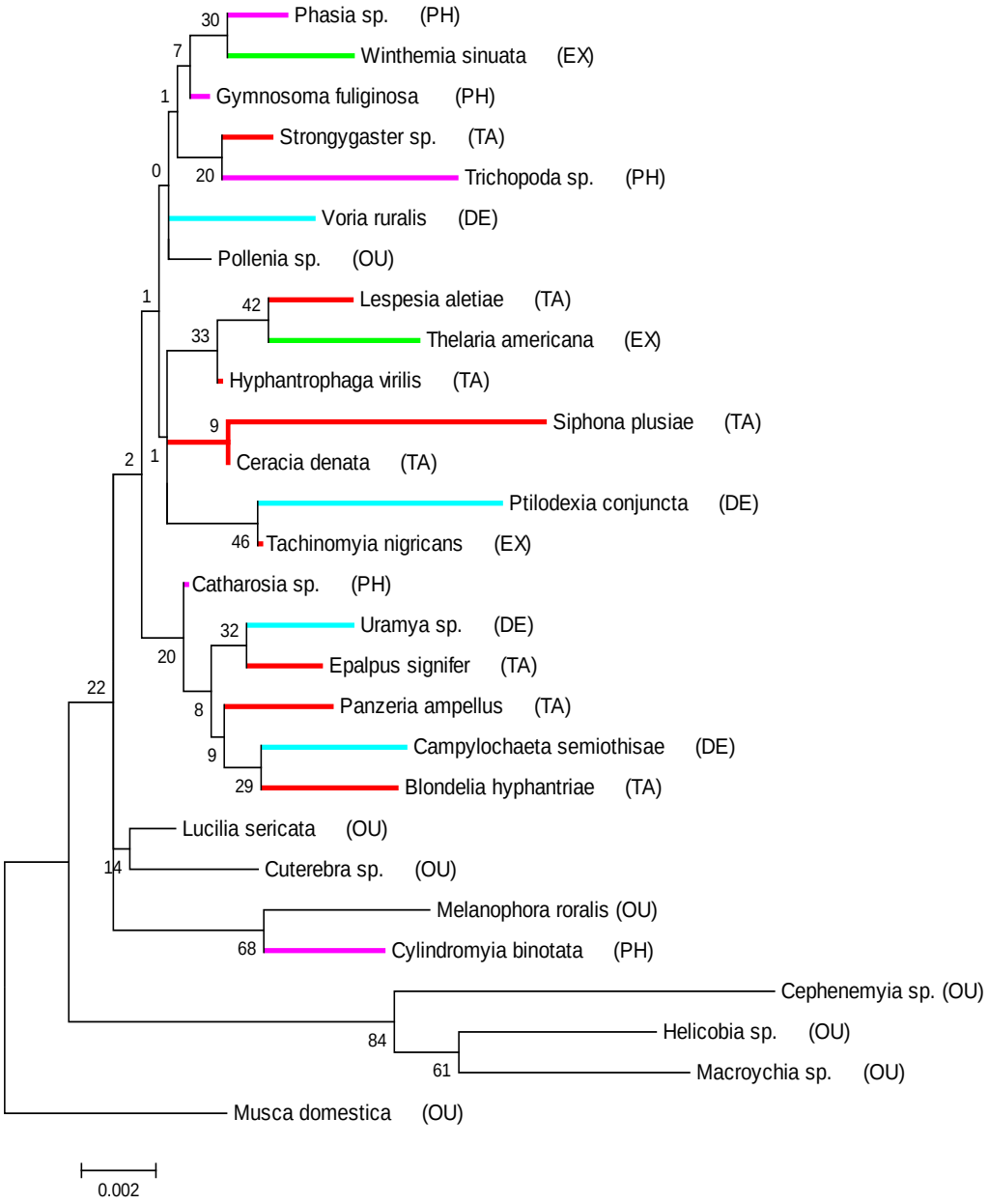

Figure 1 – Maximum likelihood tree for the 18S gene using the T92+G+I model and 1000 bootstraps. The bootstrap support values are placed to the left of each node. The branches are colored as follows: Tachininae: red; Exoristinae: green; Phasiinae: fuchsia; Dexiinae, cyan. All non-tachinids are colored black. The subfamilies are identified as follows: Dexiinae(DE), Exoristinae(EX), Phasiinae(PH), Tachininae(TA), and all others were considered outgroups (OU)

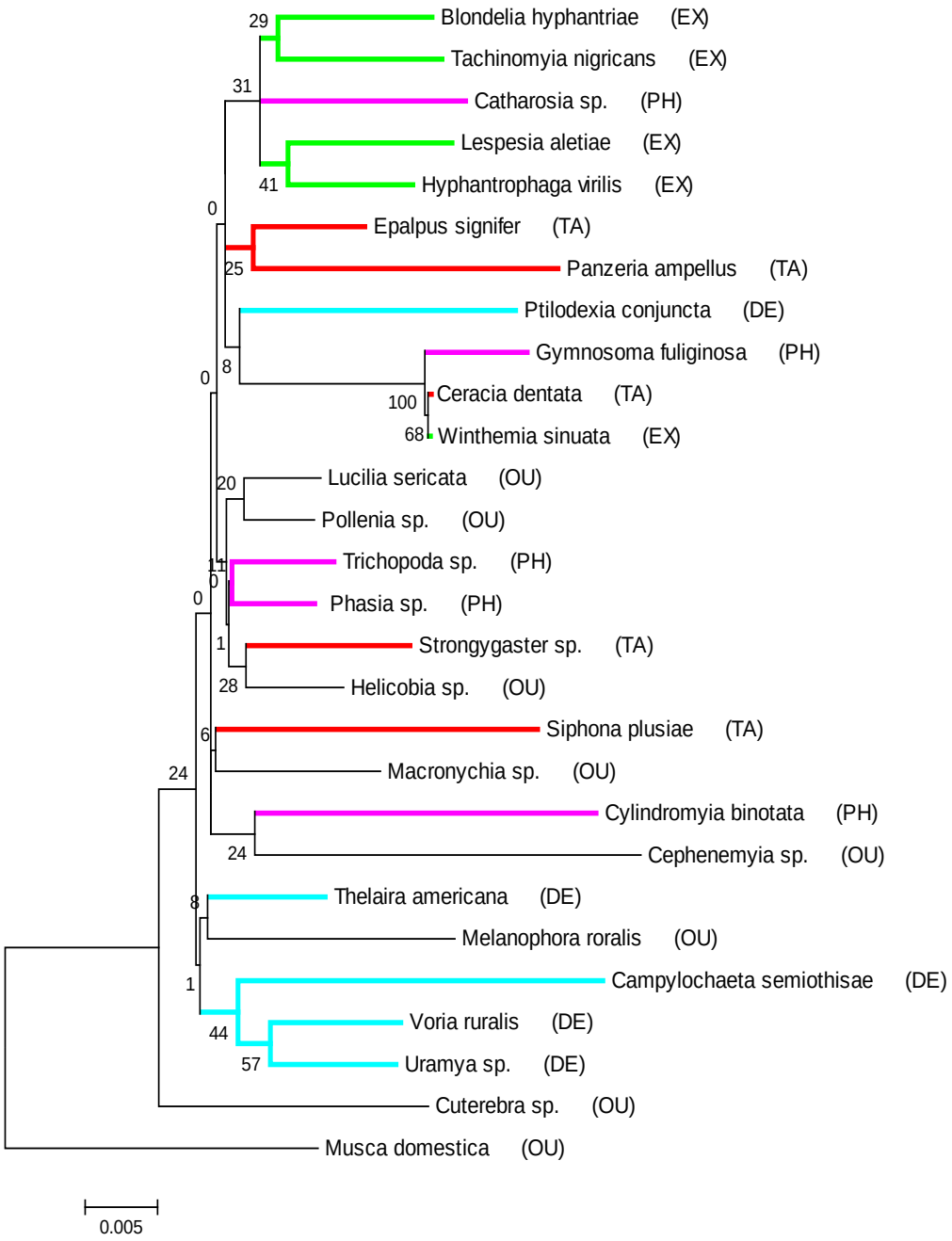

Figure 2 – Maximum likelihood tree for the 28S gene using the GTR+G+I model and 1000 bootstraps. Subfamily coloration and abbreviations are the same as in Figure 1.

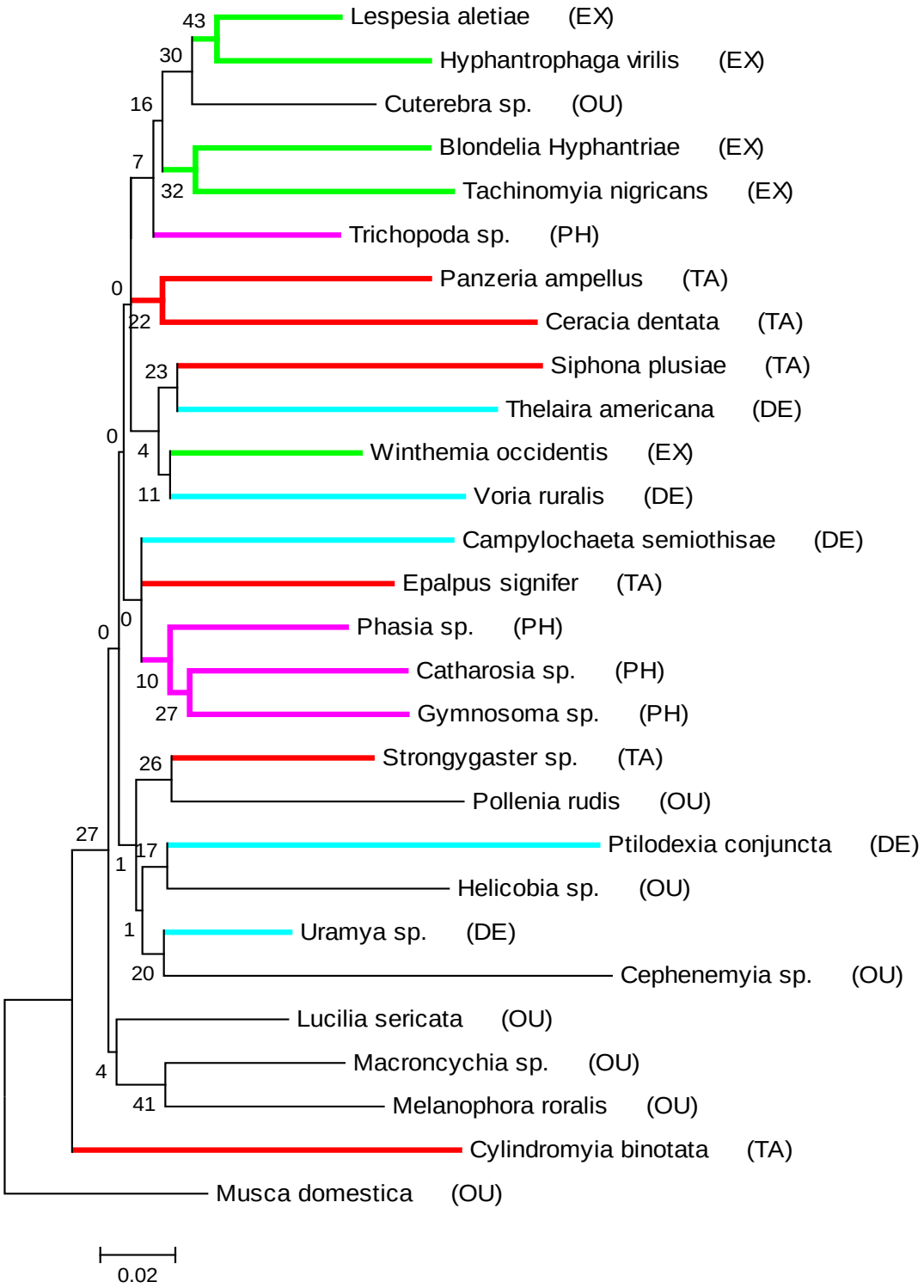

Figure 3 – Maximum likelihood tree for the COI gene using the GTR+G+I model and 1000 bootstraps. Subfamily coloration and abbreviations are the same as in Figure 1.

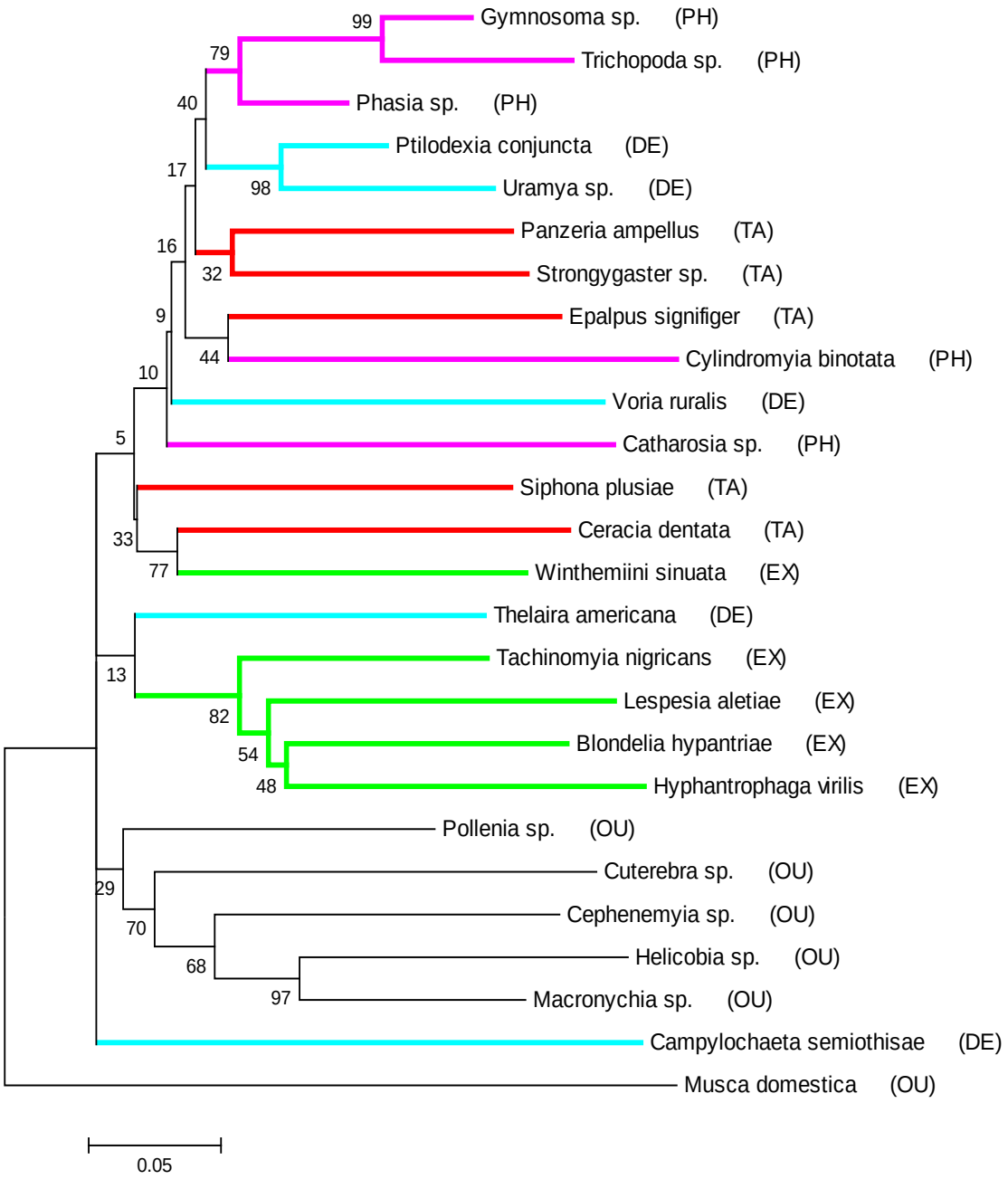

Figure 4 – Maximum likelihood tree for the CAD gene using the T92+G+I model and 1000 bootstraps. For the treatment of missing data, all sites were used compared to partial deletion for the other ML gene trees. Subfamily coloration and abbreviations are the same as in Figure 1.

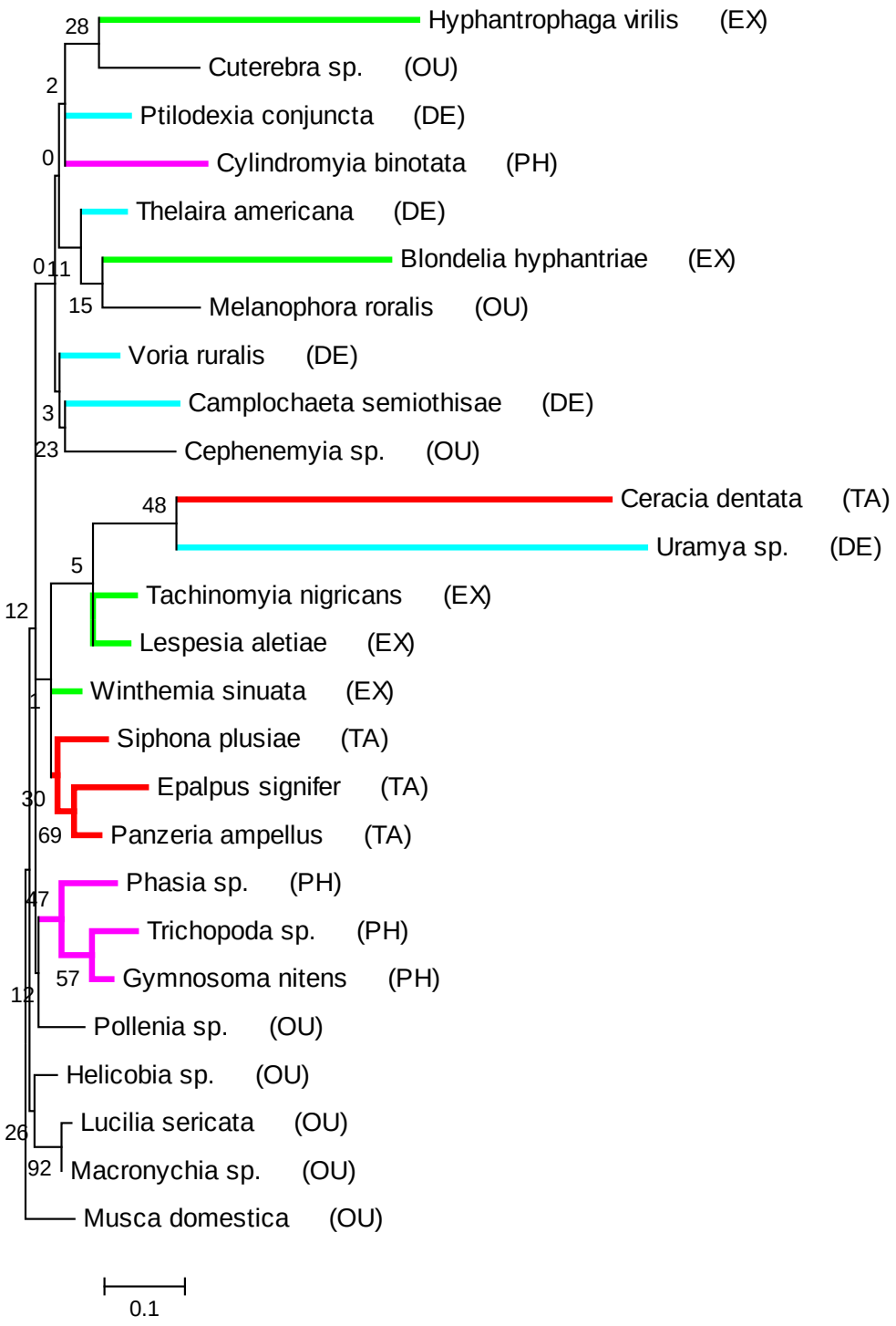

Figure 5 – Maximum likelihood tree for the EF1α gene using the GTR+G model and 1000 bootstraps. Subfamily coloration and abbreviations are the same as in Figure 1.

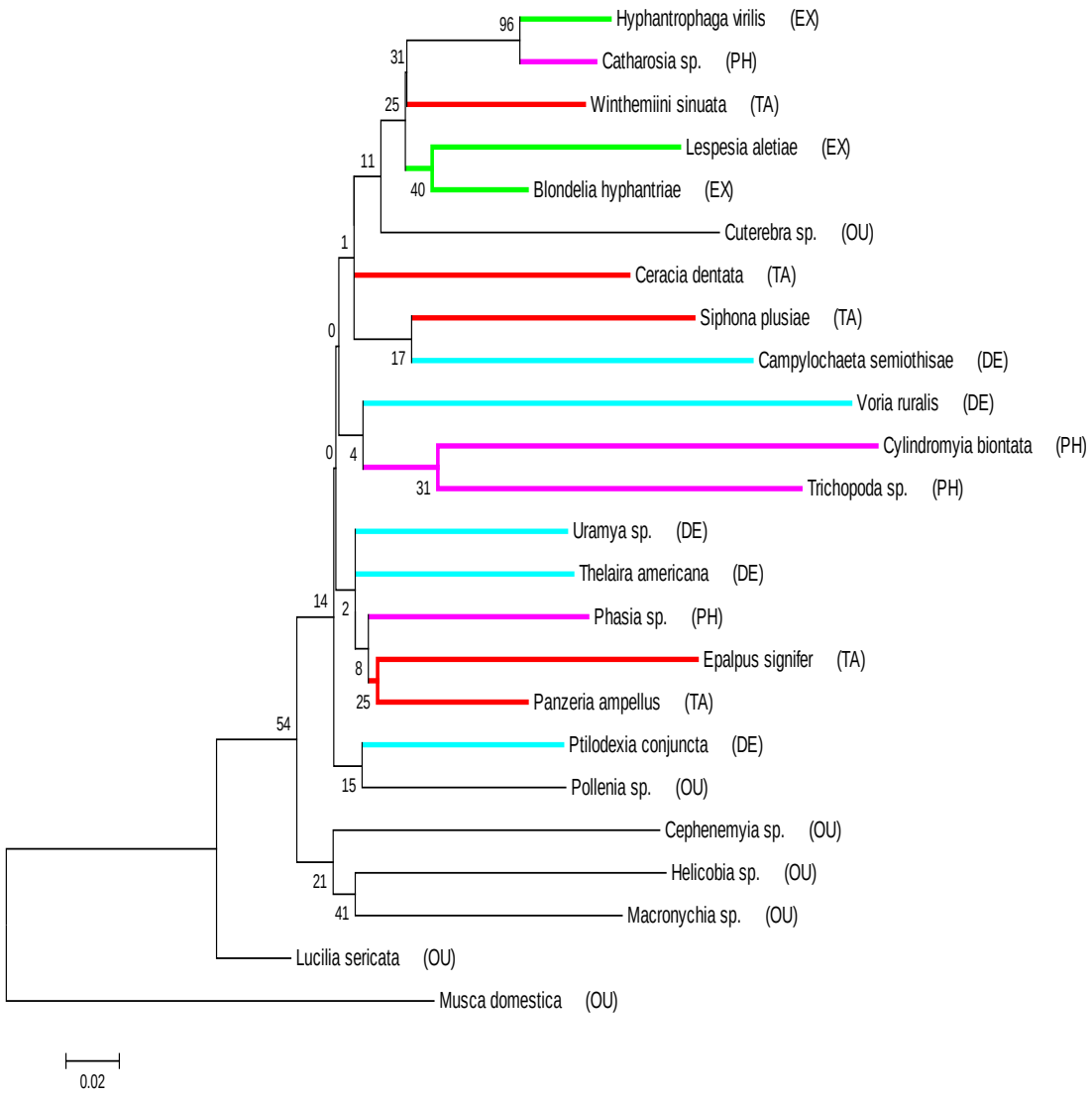

Figure 6 – Maximum likelihood tree for the TPI gene using the T92+G model and 1000 bootstraps. Subfamily coloration and abbreviations are the same as in Figure 1.

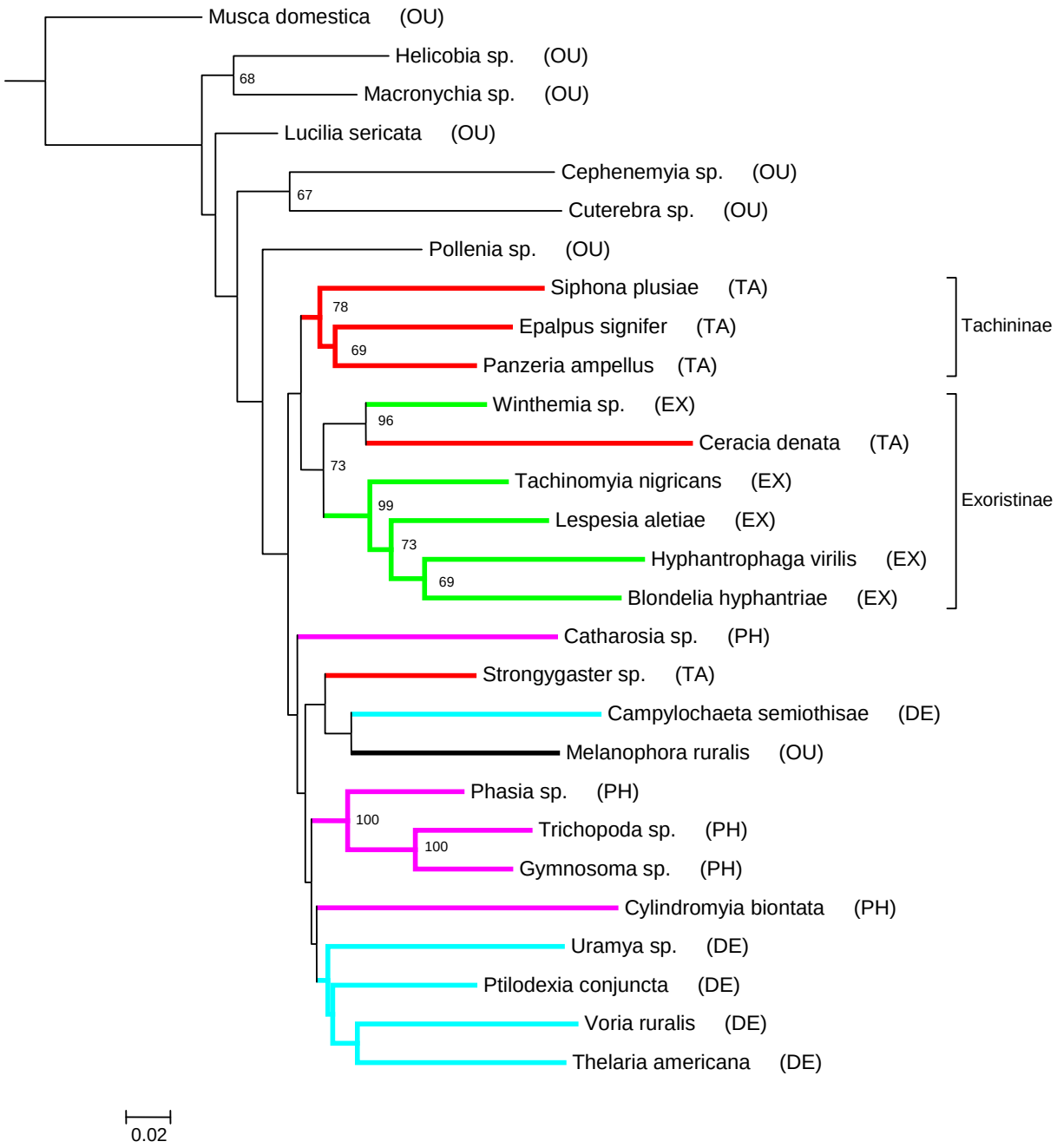

Figure 7 – GARLI Results from the concatenated gene set using the maximum likelihood criterion. Sarcophagidae, Oestridae, Tachininae, and Exoristinae (with *Ceracia*) were monophyletic. All bootstrap values under 50% were not reported. Subfamily coloration and abbreviations are the same as in Figure 1.

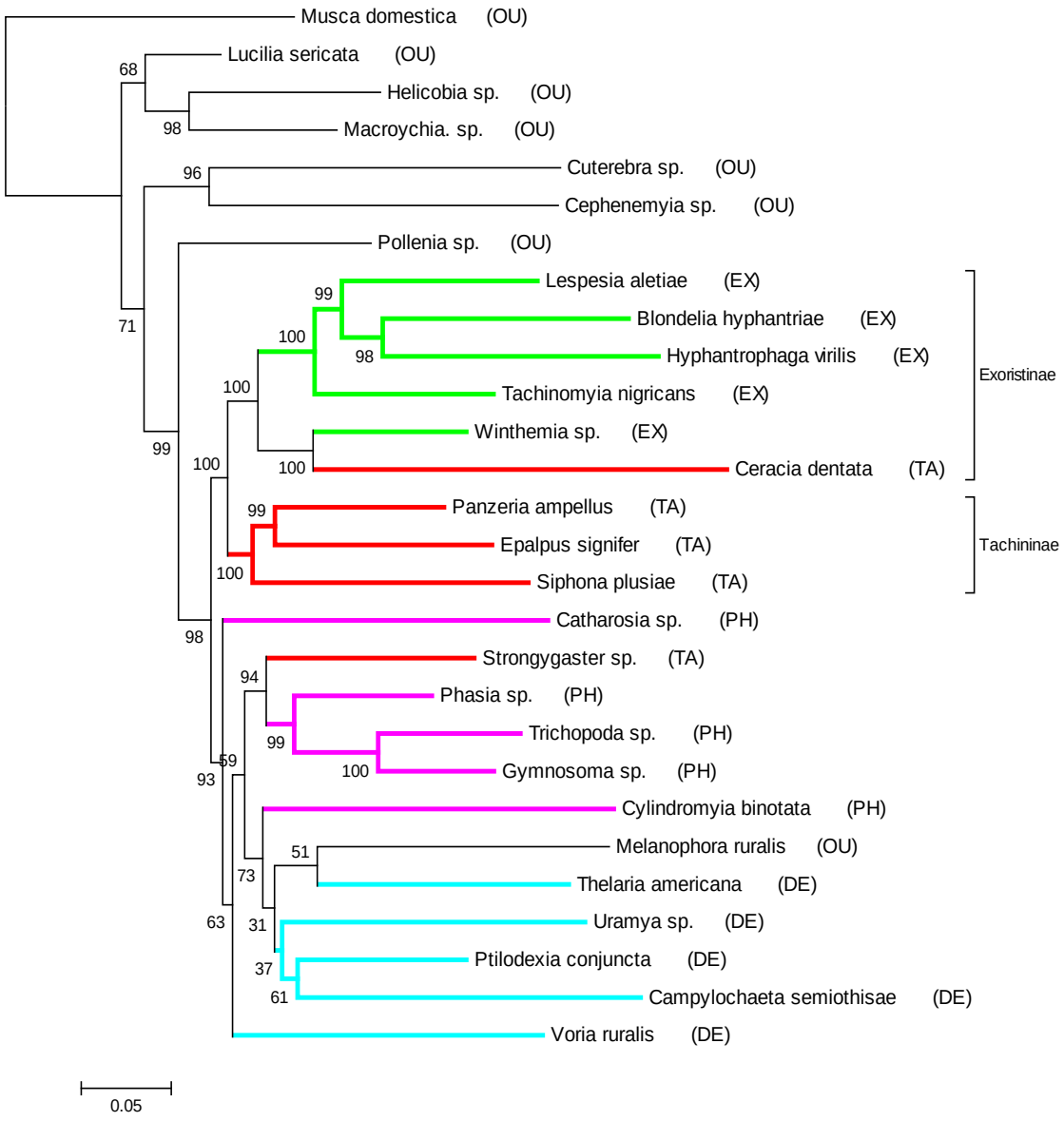

Figure 8 – An unmodified analysis using MrBayes and the entire data set. There were high support values at the basal sections of the tachinid lineage even though *Melanophora* was embedded within the Dexiinae. The posterior probabilities as a percentage lie to the left of each node. Subfamily coloration and abbreviations are the same as in Figure 1.

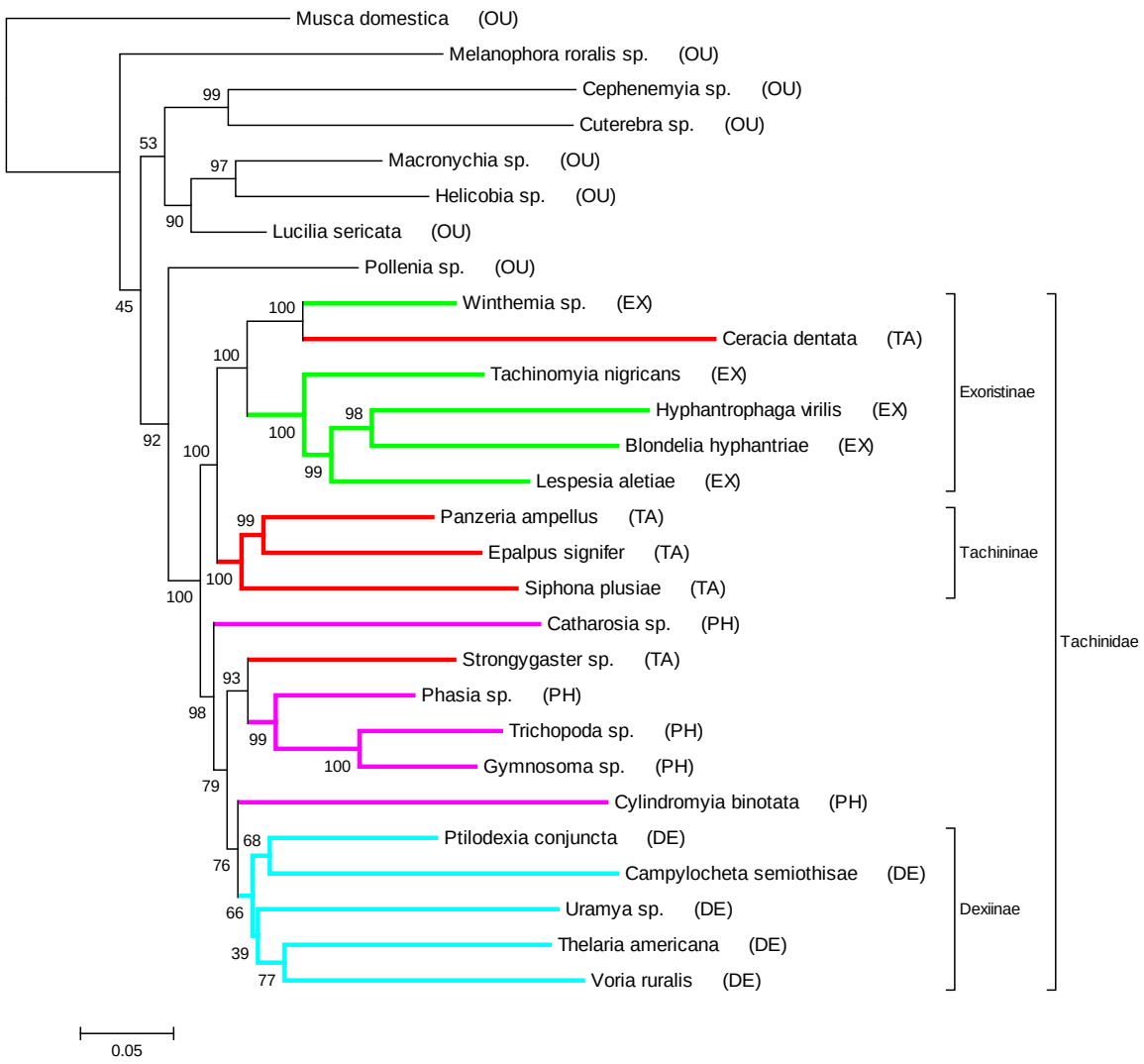

Figure 9 – A modified analysis using MrBayes which restricted all of the tachinids to be within a monophyletic group. *Melanophora* moved to the far outside of Oestroidea and created a monophyletic Dexiinae. The posterior probabilities as a percentage lie to the left of each node. Subfamily coloration and abbreviations are the same as in Figure 1.

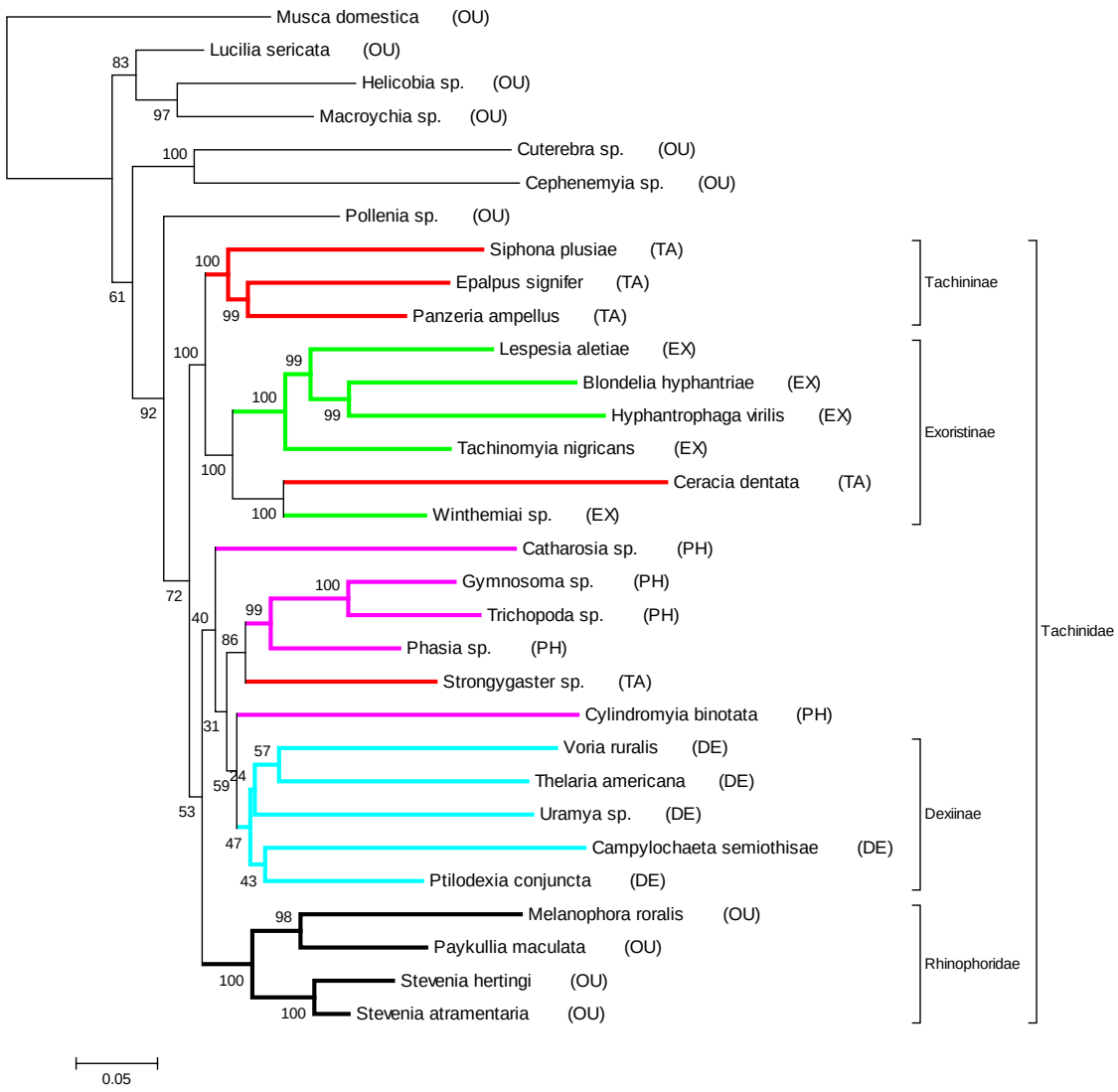

Figure 10 – An unrestricted analysis using MrBayes with additional rhinophorid sequences. The Rhinophoridae were monophyletic and moved outside of a monophyletic Dexiinae, basal to the rest of the Phasiinae(Dexiinae) clade. The posterior probabilities as a percentage lie to the left of each node. Subfamily coloration and abbreviations are the same as in Figure 1

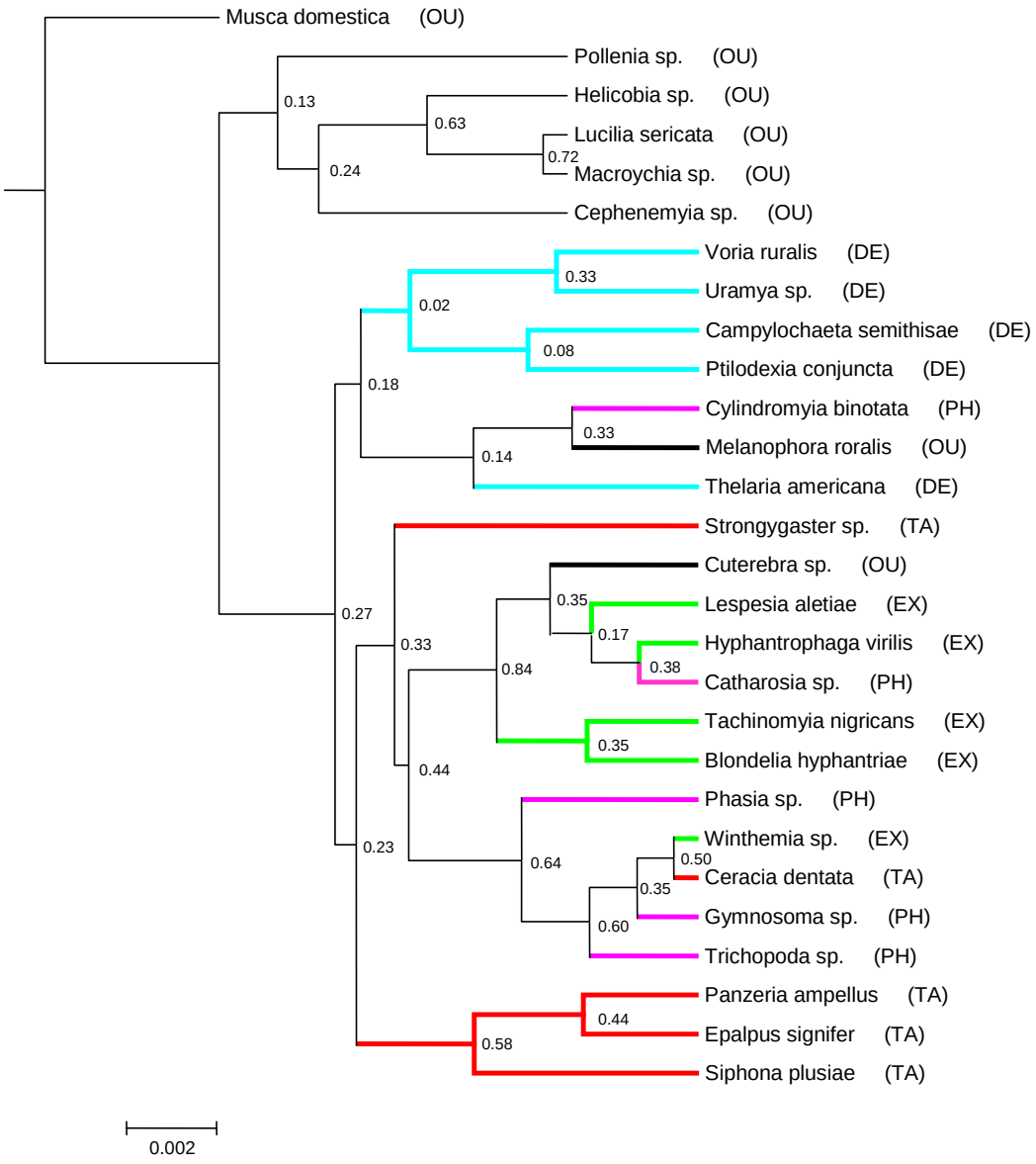

Figure 11– BEAST results using the concatenated data set under Bayesian inference. Substitution models (Table 3) were chosen using a model test in MEGA 5. The posterior probabilities lie to the right of their respective nodes. This tree was aesthetically modified using FigTree (Rambaut, 2012). Subfamily coloration and abbreviations are the same as in Figure 1.

TABLES

Table 1 – Tachinidae gene coverage and the samples used during the study. A total of 172 sequences were obtained while GenBank was used to fill in additional taxa and genes for a total of 93% gene coverage. The subfamilies are identified as follows: Dexiinae(DE), Exoristinae(EX), Phasiinae(PH), and Tachininae(TA).

Subfamily	Species	18S	28S	COI	SCAD	MCAD	EF1α	TPI
De	*Campylochaeta semiothisae*	182	182	416		207	207	182
De	*Ptilodexia conjuncta*	236	236	236	236	081	081	236
De	*Thelaira americana*	492	492	492	492	492	492	492
De	*Uramya sp.*	290	290	290	290	290	290	290
De	*Voria ruralis*	009	009	253		253	253	009
Ex	*Blondelia hyphantriae*	173	173	173	173	173	173	173
Ex	*Hyphantrophaga virilis*	363	363	282	363	363	363	363
Ex	*Lespesia aletiae*	056	056	056	056	056	056	056
Ex	*Tachinomyia nigricans*	413	413	274	413		413	
Ex	*Winthemia sinuata*	433	433	107	433	433	433	433
Ph	*Catharosia sp.*	493	493	481	481	481		481
Ph	*Cylindromyia binotata*	426	426	426	426	426	426	426
Ph	*Gymnosoma fuliginosa*	151	151	482	214	214	GQ409462	
Ph	*Phasia sp.*	164	529	529	529	529	529	529
Ph	*Trichopoda sp.*	473	473	473	473	473	473	473
Ta	*Ceracia dentata*	378	378	425	425	425	378	425
Ta	*Epalpus signifer*	122	122	122	121	419	419	122
Ta	*Panzeria ampellus*	287	287	283	287	287	287	287
Ta	*Siphona plusiae*	013	013	030	013	013	013	013
Ta	*Strongygaster sp.*	475	475	475	475			

Table 2 – Out group gene coverage and the samples used during the study. This table also includes the additional rhinophorids (*Paykullia* and two *Stevenia*) used for the third MrBayes analysis. A total of 172 sequences were obtained while GenBank was used to fill in additional taxa and genes for a total of 93% gene coverage. The outgroups were given the subfamily identifier (OU).

Subfamily	Species	18S	28S	COI	SCAD	MCAD	EF1a	TPI
Ou	*Lucilia sericata*	476	476	EU815025.1			476	476
Ou	*Macronychia sp.*	480	480	480	480	480	480	480
Ou	*Cuterebra sp.*	496	496	496	496	496	496	496
Ou	*Cephenmyia sp.*	508	508	508	508	508	508	508
Ou	*Pollenia sp.*	477	477	FR719179.1	477	477	477	477
Ou	*Melanophora ruralis*	528	528	528			528	
Ou	*Helicobia sp.*	479	479	479	479	479	479	479
Ou	*Musca Domestica*	DQ133074.1	AJ551427.1	EU815009.1	AY280689.1		AF503149.1	GQ265639.1
Ou	*Paykullia maculata*	FJ025496.1		FJ025646.2			FJ025694.1	
Ou	*Stevenia hertingi*	GQ409221.1		GQ409282.1			GQ409493.1	
Ou	*Stevenia atramentaria*	GQ409220.1	GQ409281.1				GQ409492.1	

Table 3 - This table includes information on the nucleotide substitution models used for the maximum likelihood analysis, BEAST analysis, the location of the start and ending of gene sequences in the concatenated data set, and the total number of base pairs for each gene.

Species	**18S**	**28S**	**COI**	**SCAD**	**MCAD**	**EF1a**	**TPI**
Model Used ML	T92+G+I	GTR+G+I	GTR+G+I	T92+G+I	T92+G+I	GTR+G	T92+G
Model Used BEAST	HKY+I	GTR+G+I	GTR+G+I	HKY+G+I	HKY+G+I	GTR+G	HKY+G
Concatenated Start	1	809	2366	3055	3055	4761	5697
Concatenated End	808	2365	3054	4760	4760	5696	6214
Number of Base Pairs	807	1556	688	1705	1705	935	517

Table 4 – Primers and sources of primers that were used in this study. Over 100 primer pairs were attempted but only these sets consistently amplified the target genes and were later sequenced. The CAD primers were designed by Dr. John O. Stireman based on Moulton and Wiegmann (2004).

Gene	Information
18S	(Kutty et al., 2010)
18SF	CATATCCGAGGCCCTGTAAT
18SR	AGTTTTCCCGTGTTGAGTCA
28S	(Stireman, 2002)
28SF2	CTAACAAGGATTTTCTTAGTAGCGGCGAG
28SR2	GGTGAGTTGTTACACACTCCTTAGCGGAT
COI	(Folmer et al., 1994; Smith et al., 2006)
LCO 1490	GGTCAACAAATCATAAAGATATTGG
LepR1	TAAACTTCTGGATGTCCAAAAAATCA
SCAD	(Stireman, unpublished data; Moulton and Wiegmann, 2004)
SCAD320F	RTKTTTGGTATTTGYYTGGGTCAYCA
SCAD680R	AARGCATCWCKYACYACYTCGTAYTC
MCAD	(Stireman, unpublished data; Moulton and Wiegmann, 2004)
MCAD054F	GTNGTNTTYCARACNGGNATGGT
MCAD405R	GCNGTRTGYTCNGGRTGRAAYTG
EF1a	(Stireman, 2002)
efs175 (ef1a-a)	GGAAATGGGAAAAGGCTCCTTCAAGTAYGCYTGGG
Ef2	AACTAACGGTGTGACGAGTGTA
TPI	(Weigmann et al., 2008)
M13 tpi 111Fb	TGTAAAACGACGGCCAGTGGNAAYTGGAARATGAAYGG
M13 TpiR275	CAGGAAACAGCTATGACGCCCANACNGGYTCRTANGC

Table 5 – The standard touchdown PCR program used during the study. Although several programs were used, this one produced the most consistent DNA amplifications.

Step	Temp	Time
1	94	4:00
2	94	0:30
3	55	0:30
4	72	2:00
5	Goto 2, 4 times	
6	94	0:30
7	55	1:00
8	72	2:00
9	Goto 6, 6 times	
10	94	0:30
11	50	0:20
12	72	2:30
13	Goto10, 36 times	
14	72	3:00
15	4	Hold
16	end.	

APPENDIX I

```
begin mrbayes;

  set autoclose = yes nowarn = yes;
  log start filename = mytachs.log replace;
        CHARSET 18S  =  1 -808;
        CHARSET 28S  =  809 -2365;
        CHARSET COI  =  2366 -3054;
        CHARSET CAD  =  3055 -4760;
        CHARSET EF1a =  4761 -5696;
        CHARSET TPI  =  5697 -6214;

outgroup Various.GenBank.Musca.domestica.Ou;

partition genes = 6: 18S , 28S , COI , CAD , EF1a , TPI;

set partition = genes;
        lset applyto = ( all ) nst = 6 rates = invgamma;
        unlink statefreq = ( all ) revmat = ( all ) shape = ( all ) pinvar = ( all );
        prset applyto = ( all ) ratepr = variable;

constraint tachinidae -1 = 1-20;
prset topologypr = constraints(tachinidae);

showmodel;
        mcmcp nruns = 2 ngen = 3000000 printfreq = 1000 samplefreq = 1000
nchains = 4 savebrlens = yes filename=mytachs relburnin = yes burninfrac= 0.5
Mcmcdiagn= yes Diagnfreq= 1000;

        mcmc;
        showmodel;
        sump filename=mytachs burnin= 1500 printtofile = yes;

        sumt filename=mytachs burnin= 1500 contype = allcompat;

        plot filename=mytachs parameter = lnL;

 log stop;

END;
```

REFERENCES

Abrahamson, W. G., & Blair, C. P. (2008). Sequential Radation through Host-Race Formation: Herbivore Diversity Leads to Diversity in Natural Enemies. In K. J. Tilmon (Ed.) *Specialization, Speciation, and Radiation* (pp. 188–202). Los Angeles, California: University of California Press.

Archibald, J. K., Mort, M. E., & Crawford, D. J. (2003). Bayesian Inference of Phylogeny: A Non-Technical Primer. *Taxon*, 52(2), 187-191.

Arnaud, P. J. (1958). The Entomological Publications of Charles Henry Tyler Townsend (1863-1944); With Lists of his New Generic and Specific Names. *Microentomology*, 23, 1–63.

Baker, W. A., Bradley, W. G., & Clark, C. A. (1949). Biological control of the European corn borer in the United States. *Technical Bulletin* (983), 1–185. USDA.

Belshaw, R. (1994). Life history characteristics of Tachinidae (Diptera) and their effects on polyphagy. In B. Hawkins & W. Sheehan (Eds.), *Parasitoid Community Ecology* (Vol. 24, pp. 1752–1753). USA: Oxford University Press.

Boettner, G. H., Elkinton, J. S., & Boettner, C. J. (2000). Effects of a Biological Control Introduction on Three Non-target Native Species of Saturniid Moths. *Conservation Biology*, 14(6), 1798–1806.

Cerretti, P. (2010). *I tachinidi della fauna italiana (Diptera Tachinidae), con chiave interattiva dei generi ovest-paleartici*.Verona, Italy: Cierre Edizioni.

Coombs, G., Dold, A. P., & Peter, C. I. (2011). Generalized fly-pollination in *Ceropegia ampliata* (Apocynaceae–Asclepiadoideae): the role of trapping hairs in pollen export and receipt. *Plant Systematics and Evolution*, 296, 137–148.

Cooper, T. M., Frank, J. H., Cave, R. D., Burton, M. S., Dawson, J. S., & Smith, B. W. (2011). Release and monitoring of a potential biological control agent, *Lixadmontia franki*, to control an invasive bromeliad-eating weevil, *Metamasius callizona*, in Florida. *Biological Control*, 59, 319–325.

Crosskey, R. W. (1976). A Taxonomic Conspectus of the Tachinidae (Diptera) of the Oriental Region. Bulletin of the British Museum (Natural History) Entomology Supplement 26. 1-357.

Crosskey, R. W. (1977). Family Tachinidae. In M. D. Delfinado & D. E. Hardy (Eds.), *A Catalog of the Diptera of the Oriental Region* (pp. 586–697). Honolulu, HI: The University Press of Hawaii.

Crosskey, R. W. (1980). Family Tachindae. In R. W. Crosskey (Ed.) *Catalogue of the Diptera of the Afrotropical region* (pp. 822–882). London: British Museum (Natural History).

De Bach, P. (1974). *Biological control by natural enemies*. New York: Cambridge University Press.

Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology.* 7(214), 1-8.

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian Phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29(8), 1969–1973.

Eggleton, P., & Belshaw, R. (1992). Insect Parasitoids: An Evolutionary Overview. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 337(1279), 1–20.

Eggleton, P., & Belshaw, R. (1993). Comparison of dipteran, hymenopteran and coleopteran parasitoids: provisional phylogenetic explanations. *Biological Journal of the Linnean Society*, 48, 213–226.

Feder, J. L., & Forbes, A. A. (2010). Sequential speciation and the diversity of parasitic insects. *Ecological Entomology*, 35, 67–76.

Feener, D. H., & Brown, B. V. (1997). Diptera as parasitoids. *Annual Review of Entomology*, 42, 73–97.

Felsenstein, J. (2004). *Inferring Phylogenies*. Sunderland, Massachusetts: Sinauer Associates, Inc.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3(5), 294–299.

Gibson, J. F., Kelso, S., Jackson, M. D., Kits, J. H., Miranda, G. F. G., & Skevington, J. H. (2011). Diptera-Specific Polymerase Chain Reaction

Amplification Primers of Use in Molecular Phylogenetic Research. *Annals of the Entomological Society of America*, 104(5), 976-997.

Godfray, H. (1994). *Parasitoids: behavioral and evolutionary ecology*. Princeton, NJ: Princeton University Press

Grenier, S. (1988). Applied biological control with Tachinid flies (Diptera, Tachinidae): A review. *Anzeiger für Schädlingskunde Pflanzenschutz Umweltschutz*, 61(3), 49–56.

Griffin, A., Hingston, A., & Ohmart, C. (2009). Pollinators of *Eucalyptus regnans* (Myrtaceae), the world's tallest flowering plant species. *Australian Journal of Botany*, *57*, 18–25.

Gross, P. (1993). Insect Behavioral and Morphological Defenses Against Parasitoids. *Annual Review of Entomology*, 38, 251–273.

Hall, B. G. (2007). *Phylogenetic trees made easy: a how-to manual* (3rd ed.). Sunderland, Massachusetts: Sinauer Associates, Inc.

Herting, B. (1960). Biology of the West Palearctic Tachids (Dipt. Tachinidae). *Monographs on Applied Entomology*, 16, 5–32.

Herting, B. (1984). Catalogue of Palearctic Tachinidae. *Stuutgarter Beitrage zur Naturkunde Serie A*, 369, 1–228.

Huelsenbeck, J. P., & Ronquist, F. (2001) MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics.* 17(8), 754-755.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R., & Bollback, J. P. (2001). Bayesian Inference of Phylogeny and Its Impact on Evolutionary Biology, *Science*, *294*, 2310–2314.

Irwin, M. E., Schlinger, E. I., & Thompson, F. C. (2003). Diptera, True Flies. In S. Goodman & J. Benstead (Eds.), *The Natural History of Madagascar* (pp. 692–702). London: University of Chicago Press.

Kearns, C. (1992). Anthophilous fly distribution across an elevation gradient. *American Midland Naturalist*, *127*, 172–182.

Kellogg, S., Fink, L., & Brower, L. (2003). Parasitism of native luna moths, *Actias luna* (L.) (Lepidoptera: Saturniidae) by the introduced *Compsilura concinnata* (Meigen) (Diptera: Tachinidae) in central Virginia, and their hyperparasitism by trigonalid wasps (Hymenoptera: Trigonalidae) *Environmental entomology*, 32(5), 1019–1027.

Kimberling, D. N., Miller, J. C., & Penrose, R. L. (1986). Distribution and parasitism of witner moth, *Operophtera brumata* (Lepidoptera: Geometridae), in western Oregon. *Enviormental Entomology*, 15(5), 1042–1046.

Kutty, S., Bernasconi, M., & Sifner, F. (2007). Sensitivity analysis, molecular systematics and natural history evolution of Scathophagidae (Diptera: Cyclorrhapha: Calyptratae). *Cladistics*, 23, 64-83.

Kutty, S., Pape, T., Pont, A., Wiegmann, B. M., & Meier, R. (2008). The Muscoidea (Diptera: Calyptratae) are paraphyletic: Evidence from four mitochondrial and four nuclear genes. *Molecular phylogenetics and evolution*, 49, 639–652.

Kutty, S., Pape, T., Wiegmann, B. M., & Meier, R. (2010). Molecular phylogeny of the Calyptratae (Diptera: Cyclorrhapha) with an emphasis on the superfamily Oestroidea and the position of Mystacinobiidae and McAlpine's fly. *Systematic Entomology*, *35*, 614–635.

Lehnebach, C. A, & Robertson, A. W. (2004). Pollination ecology of four epiphytic orchids of New Zealand. *Annals of Botany*, *93*(6), 773–81.

Liebhold, A., Halverson, J., & Elmes, G. (1992). Gypsy moth invasion in North America: a quantitative analysis. *Journal of Biogeography*, 19(5), 513–520.

Lorenz, R., Bernhart, S. H., Höner Zu Siederdissen, C., Tafer, H., Flamm, C., Stadler, P. F., & Hofacker, I. L. (2011). ViennaRNA Package 2.0. *Algorithms for Molecular Biology,* 6(26), 1-14.

Louda, S. M., Pemberton, R. W., Johnson, M. T., & Follett, P. A. (2003). Nontarget effects--the Achilles' heel of biological control? Retrospective analyses to reduce risk associated with biocontrol introductions. *Annual Review of Entomology*, 48, 365–396.

Maddison, W. P., Knowles, L. L. (2006) Inferring phylogney despite incomplete linage sorting. *Systematic Biology*, 55(1), 21-30.

Marinho, M. A. T., Junqueira, A. C. M., Paulo, D. F., Esposito, M. C., Villet, M. H., & Azeredo-Espin, A. M. L. (2012). Molecular phylogenetics of Oestroidea (Diptera: Calyptratae) with emphasis on Calliphoridae: Insights into the inter-familial relationships and additional evidence for paraphyly among blowflies. *Molecular Phylogenetics and Evolution*, 65, 840–854.

McAlpine, J. F. (1989). Phylogeny and Classification of the Muscomorpha. In J. F. McAlpine & D. M. Wood (Eds.) *Manual of Nearctic Diptera Volume 3*. (pp. 1397-1502). Ottawa, Ontario: Research Branch Agriculture Canada.

McManus, M., Schneeberger, N., Reardon, R., & Mason, G. (1992). Gypsy Moth. *Forest Insect & Disease Leaflet* (162). USDA. 1-13.

Moulton, J. K., & Wiegmann, B. M. (2004). Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). *Molecular Phylogenetics and Evolution*, 31, 363–378.

Myers, J. H., Savoie, A., & Van Randen, E. (1998). Eradication and pest management. *Annual Review of Entomology*, 43, 471–491.

Nirmala, X., Hypsa, V., & Zurovec, M. (2001). Molecular phylogeny of Calyptratae (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNAs in higher dipterans and their use in phylogenetic inference. *Insect Molecular Biology*, 10(5), 475–485.

O'Hara, J. E. (2010). World genera of the Tachinidae (Diptera) and their regional occurrence. Available from http://www.nadsdiptera.org/Tach

O'Hara, J. E., & Wood, D. M. (2004). Catalogue of the Tachinidae (Diptera) of America north of Mexico. *Memoirs on Entomology, International*, *18*.

Palumbi, S. R. (1996). Nucleic Acids II: The Polymerase Chain Reaction. In D. M. Hillis, C. Moritz, & B. K. Mable (Eds.), *Molecular Systematics* (2nd ed., pp. 205–247). Sunderland, Massachusetts: Sinauer Associates, Inc.

Pape, T. (1986). A phylogenetic analysis of the woodlouse-flies (Diptera, Rhinophoridae). *Tijdschrift voor Entomologie*, 129, 15–34.

Pape, Thomas. (2010). Rhinophoridae (Woodlouse Flies). In B. V. Brown, A. Borkent, J. M. Cumming, D. M. Wood, N. E. Woodley, & M. A. Zumbado (Eds.), *Manual of Central American Diptera* (pp. 1337–1341). Ottawa: National Research Council of Canada.

Petersen, F. T., Meier, R., Kutty, S. N., & Wiegmann, B. M. (2007). The phylogeny and evolution of host choice in the Hippoboscoidea (Diptera) as reconstructed using four molecular markers. *Molecular Phylogenetics and Evolution*, 45, 111–122.

Pruitt, K. D., Tatusova, T., Brown, G. R., & Maglott, D. R. (2012). NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. *Nucleic Acids Research*, 40(Database issue), D130–135.

Rambaut, A. (2012). FigTree. [software] University of Edinburgh. Available from http://tree.bio.ed.ac.uk/software/figtree/

Rasnitsyn, A. P. (1988). An outline of evolution of the hymenopterous insects. *Oriental Insects*, (22), 115–145.

Rognes, K. (1986). The systematic position of the genus Helicobosca Bezzi with a discussion of the monophyly of the calyptrate families Calliphoridae, Rhinophoridae, Sarcophagidae. *Entomologica Scandinavica*, 7, 75–92.

Rognes, K. (1997). The Calliphoridae (Blowflies) (Diptera: Oestroidea) are not a monophyletic Group,. *Cladistics*, 13, 27–66.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19(12), 1572–1574.

Sabrosky, C. W., & Arnaud, P. H. J. (1965). Family Tachinidae. *A Catalog of the Diptera of America North of Mexico* (pp. 967–1108). Washington, D.C.: USDA.

Sassaman, C., & Garthwaite, R. (1984). The interaction between the terrestrial isopod *Porcellio scaber* Latreille and one of its dipteran parasites, *Melanophora roralis* (L.)(Rhinophoridae). *Journal of Crustacean Biology*, 4(4), 595–603.

Shewell, G. (1987). Sarcophagidae. In J. F. McAlpine (Ed.), *Manual of Nearctic Diptera* (pp. 1159–1184). Ottawa: Agriculture Canada.

Shima, H. (1989). Parasitic way of life in Tachinid flies. *Insectarium*, *26*. 1-26.

Smith, M. A., Wood, D. M., Janzen, D. H., Hallwachs, W., & Hebert, P. D. N. (2007). DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceedings of the National Academy of Sciences*, 104(12), 4967–4972.

Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of the National Academy of Sciences*, 103(10), 3657–3662.

Stireman, J O. (2005). The evolution of generalization? Parasitoid flies and the perils of inferring host range evolution from phylogenies. *Journal of Evolutionary Biology*, 18, 325–336.

Stireman, John O, O'Hara, J. E., & Wood, D. M. (2006). Tachinidae: evolution, behavior, and ecology. *Annual Review of Entomology*, 51, 525–555.

Stireman, John O. (2002). Phylogenetic relationships of tachinid flies in subfamily Exoristinae (Tachinidae: Diptera) based on 28S rDNA and elongation factor-1alpha. *Systematic Entomology*, 27, 409–435.

Stireman, John O., & Singer, M. (2003). Determinants of parasitoid-host associations: insights from a natural tachinid-lepidopteran community. *Ecology*, 84(2), 296–310.

Stocsits, R. R., Letsch, H., Hertel, J., Misof, B., & Stadler, P. F. (2009). Accurate and efficient reconstruction of deep phylogenies from structured RNAs. *Nucleic Acids Research*, 37(18), 6184–6193.

Swofford, D. L., Olsen, G. J., Waddell, P. J., & Hillis, D. M. (1996). Phylogenetic Inference. In D. M. Hillis, C. Moritz, & B. K. Mable (Eds.), *Molecular Systematics* (2nd ed., pp. 407–514). Sunderland, Massachusetts: Sinauer Associates, Inc.

Tachi, T., & Shima, H. (2009). Molecular phylogeny of the subfamily Exoristinae (Diptera, Tachinidae), with discussions on the evolutionary history of female oviposition strategy. *Systematic Entomology*, 35, 148–163.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28(10), 2731–2739.

Torre-Bueno, J. R. (1978). *A Glossary of Entomology*. G. S. Tulloch (Ed.). New York: New York Entomological Society.

Tschorsnig, H. P. (1985). Taxonomy of important parasites in forestry: investigations on the structure of the male postabdomen of the Tachinidae (Diptera). *Stuttgarter Beitrage zur Naturkunde Serie A (Biologie)*, 383, 1–137.

Van Driesche, R. G., Carruthers, R. I., Center, T., Hoddle, M. S., Hough-Goldstein, J., Morin, L., Smith, L., et al. (2010). Classical biological control for the protection of natural ecosystems. *Biological Control*, 54, S2–S33.

Wiegmann, B. M., Trautwein, M. D., Winkler, I. S., Barr, N. B., Kim, J.-W., Lambkin, C., Bertone, M. a., et al. (2011). Episodic radiations in the fly tree of life. *Proceedings of the National Academy of Sciences*, 108(14), 5690–5695.

Wood, D. (1987). Tachinidae. In J. F. McAlpine (Ed.), *Manual of Nearctic Diptera* (pp. 1193–1269). Ottawa: Agriculture Canada.

Wood, D. M. (1987). Rhinophoridae. In J. F. McAlpine (Ed.), *Manual of nearctic Diptera* (pp. 1187–1191). Ottawa: Agriculture Canada.

Wood, D. M., & Zumbado, M. A. (2010). Tachinidae (Tachinid Flies, Parasitic Flies). In B. V. Brown, A. Borkent, J. M. Cumming, D. M. Wood, N. E.

Woodley, & M. A. Zumbado (Eds.), *Manual of Central American Diptera* (Vol. 2, pp. 1343–1417). Ottawa: National Research Council of Canada.

Yeates, D. K., Wiegmann, B. M., Courtney, G. W., Meier, R., Lambkin, C., & Pape, T. (2007). Phylogeny and systematics of Diptera: two decades of progress and prospects. *Zootaxa*, 1668, 565–590.

Yeates, D., & Wiegmann, B. (1999). Congruence and controversy: toward a higher-level phylogeny of Diptera. *Annual Review of Entomology*, 44, 397–428.

Zwickl, D. J. (2006). *Genetic Algorithm approachs for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion.*(Doctoral dissertation) The University of Texas at Austin.